University of Alberta

The role of Hyaluronan Synthase in Multiple Myeloma and Waldenstrom's Macroglobulinemia

by

©

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Abstract

In this study, we show that the hyaluronan synthase1 (HAS1) gene undergoes aberrant intronic splicing. In addition to HAS1 full length, we identified three novel splice variants of HAS1, HAS1Va, HAS1Vb and HAS1Vc, detected in malignant B cells from multiple myeloma (MM) and Waldenstrom's macroglobulinemia (WM). We demonstrate that expression of HAS1 variants is characteristic of MM and WM cells and is the first prognostic biomarker described for the circulating compartment of the MM clone. Also, MM and WM cells expressing one or more HAS1 variants synthesize extracellular and/or intracellular hyaluronan (HA). Expression of the HAS1Vb splice variant was significantly correlated with reduced survival of MM patients (p=0.005). Together, alternative and aberrant HAS1 gene splicing, the correlations between HAS1 splicing and HA synthesis, the correlations between HAS1 splicing and reduced survival of MM patients, and the expression of HAS1 intronic splice variant transcripts in the majority of WM cells, as confirmed at the single cell level support the hypothesis that the HAS1 splice variants play a significant role in progression of MM and WM. HAS1 gene sequencing analysis performed in MM and WM patients revealed an accumulation of inherited and acquired genetic variations most of which are absent from healthy donors. A significant proportion of these HAS1 mutations are recurrent which include inherited germline origin mutations and somatically acquired hematopoietic origin and tumor specific mutations. Our findings suggest that germline origin HAS1 mutations may predispose individuals to MM and WM. Acquired hematopoietic origin HAS1 mutations provide "secondary predisposing" biomarkers that are indicators of mutational events that appear to precede overt malignancy and identify individuals at higher risk of malignant

transformation. The patterns of tumor-specific mutations provide clonal markers for malignant MM and WM cells. The presence of these mutations makes possible a clinically feasible strategy for monitoring of patients to identify clinically cryptic malignant cells as well as to precisely evaluate response to treatment. The impact of hematopoietic origin mutations on HAS1 gene splicing is manifested only in the context of accompanying tumor-specific HAS1 mutations that in combination give rise to the clinically significant aberrant splicing of HAS1.

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Dedication

In loving memory of my grandfather, Grigol Ninua, who did every humanly possible thing to entangle me with science.

And

In loving memory of my mother and to her last "sacrifice" which she made and turn me into something.

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List of Abbreviations

ABI	Applied Biosystems
ARE	AU (Adenylate Uridylate) rich element
ASD	Alternative Splicing Database
β_2-M	Beta-2-Microglobulin
BBP	Branch point binding protein
B-CLL	Chronic Lymphocytic B-Leukemia
B-HABP	Biotinylated HA binding protein
BLAST	Basic Local Alignment Search Tool
BM	Bone marrow
BMCs	Bone marrow cells
BP	Branch point
bp	Base Pair
BRCA	breast cancer (gene)
cDNA	Complimentary DNA
CI	Confidence intrval
Cys	Cysteine
DAPI	4',6-Diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
ECM	Extracellular matrix
ERK	extracellular regulated kinase
ESE	Exonic splicing enhance
ESS	Exonic splicing suppressor

FAK	Focal adhesion kinase
FGFR	Fibroblast Growth Factor Receptor
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
GVs	Genetic variations
HA	Hyaluronan
HAase	Hyaluronidase
HABP1	Hyaluronan binding protein 1
HAPLN	HA binding link protein gene family
HASs	Hyluronan synthases
hCh	Human chromosome
HDs	Healthy donors
hnRNPs	Heterogeneous nuclear ribonucleoproteins
HPCs	Hematopoietic progenitor cells
HR	Hazard ration
HRAS	v-Ha-ras Harvey Rat Sarcoma viral oncogene homolog
HWE	Hardy-Weinberg equilibrium
Ig	Immunoglobulin
IgH	Immunoglobulin Heavy Chain
IL-6	Interleukin 6
ISE	intronic splicing enhance
ISS	intronic splicing suppressor
kDa	Kilo Dalton

KRAS	v-Ki-ras2 Kirsten Rat Sarcoma viral oncogene homolog
LSM	Laser Scanning Microscope
MAF	v-maf Musculoaponeurotic Fibrosarcoma oncogene homolog
MAFB	v-maf Musculoaponeurotic Fibrosarcoma oncogene homolog B
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-activated protein kinase kinase
MGUS	Monoclonal Gammopathy of Undetermined Significance
MM	Multiple Myeloma
MMPs	Metalloproteinases
MMSET	Multiple Myeloma SET domain containing protein
mRNA	Messenger Ribonucleic Acid
MT	Mutated
NCBI	National Center for Biotechnology Information
nt	Nucleotide
ORF	Open Reading Frame
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PC	Plasma cell
PCR	Polymerase Chain Reaction
PEA	Particle Exclusion Assay
PG	Proteoglycans
PKC	Protein kinase C

PLL	Poly-L-Lysine
PPT	Polypyrimidine tract
PSI-BLAST	Position Specific Iterated-BLAST from NCBI
PTB	Polypyrimidine tract binding protein
PTC	Premature termination codon
RFU	Relative fluorescence units
RHAMM	Receptor for Hyaluronan mediated motility
RNA	Ribonucleic Acid
RRM	N-terminal RNA recognition motifs
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SF	Splicing factor
SNPs	Single nucleotide polymorphisms
snRNPs	Small nuclear ribonucleoproteins
SR	Serine/arginine-rich protein family of splicing factors
SS	Splice sites
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WM	Waldenstrom's macroglobulinemia
WT	Wild type
XP	Xeroderma pigmentosum

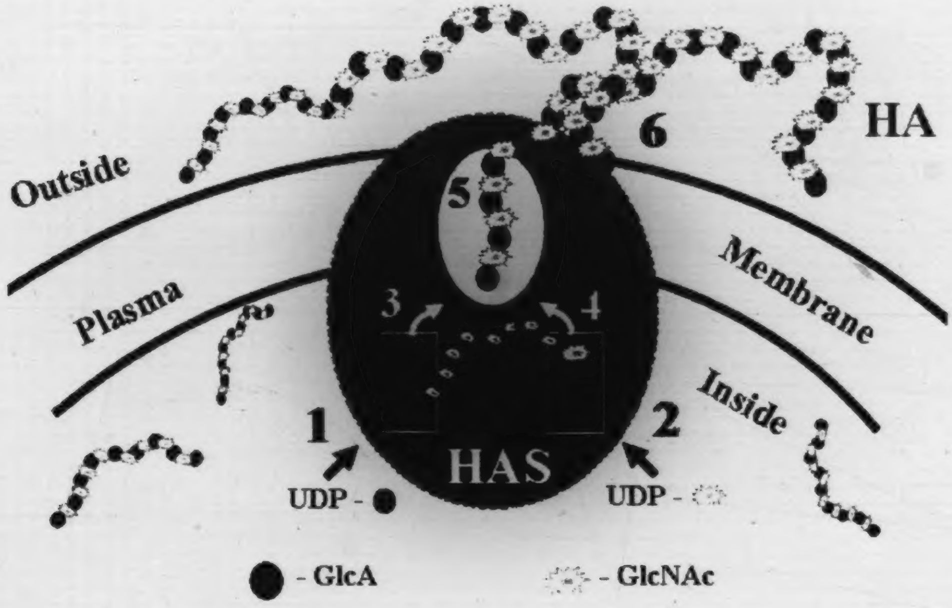
Chapter 1: Introduction

I. 1. 1. Hyaluronan synthases and their role in cancer

Hyaluronan synthases (HASs) are integral plasma membrane proteins with a number of significant functions[1-3]. HASs are unique proteins. They have two transferase activities and conduct six different functions to produce, assemble and extrude hyaluronan (HA) into the extracellular matrix (ECM) or deposit HA molecules intracellularly (Figure 1-1). The synthesis of HA by HAS proteins was not described until the 1990s. The topological structure of the HASs, proposed by Heldermon and DeAngelis, includes four transmembrane domains, two extracellular loops, two membrane-associated regions, an intracellular central loop, and intracellular amino and carboxyl terminus[1,3-6]. Examination of the hydrophobic domains of HAS isoenzymes has revealed the existence of essential cysteine (Cys) residues in the amino acid sequences of the central domain. Modification of these Cys residues changes the enzymatic activities of the HAS proteins. A significant number of protein kinase C (PKC) phosphorylation sites are predicted to occur within the intracellular loop of the HAS protein suggesting that HAS activation is perhaps regulated by direct phosphorylation.

Three isoenzymes of HAS—HAS1, HAS2, and HAS3—have been detected in humans thus far. The related but separate genes of the HASs, which share each other at least one or two exon-intron boundaries and 55-71% amino acid sequence identity, are located on different chromosomes (hCh19-*HAS1*, hCh8-*HAS2*, hCh16-*HAS3*) and encode three different proteins with distinct enzymatic properties[7,8]. These similarities in gene structure suggest that these genes might have arisen by a gene duplication event[7]. In

Figure 1-1. Hyaluronan Synthase (HAS) and its functions



The functions of HAS proteins are as follows: 1) UDP-GlcNAc (uridine diphospho-N-acetylglucosamine) binding to the HAS protein; 2) UDP-GlcA (uridine diphospho-glucose) binding to the HAS protein; 3) β(1,4) GlcNac transfer by HAS transferase activity; 4) β(1,3) GlcA transfer by HAS transferase activity; 5) Newly synthesized HA binding to HAS protein; 6) HA translocation through the membrane into the ECM.

transfectants, each isoenzyme of the HAS protein synthesizes different sizes of HA molecules with different functions[8].

HAS3, which synthesizes shorter forms of HA molecules (0.12-1x106), is thought to be more active than HAS1 and HAS2, both of which produce longer HA molecules (3.9x106)[8]. It has not yet been conclusively established whether endogenous HAS isoenzymes synthesize different sizes of HA molecules. Within *in vivo* systems, it is expected that the existence of various sizes of HA molecules result either from upregulation of hyaluronidases, which degrade the long chain of HA, or from synthesis of short HA molecules by HASs. Differential expression of HAS genes has been detected in human adult tissue by a northern analysis[7]. HAS1 and HAS3 transcripts were highly expressed in the heart, liver, skeletal muscles, prostate and ovary, while HAS2 transcripts were predominantly expressed in the heart and small intestine[7]. After the work reported in this thesis was performed, two splice variants of HAS3 transcripts were reported in the NCBI (The National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov). The functions of these HAS3 splice variants have not yet been determined.

I. 1. 2. HASs in cancer biology

A growing body of evidence in the published literature demonstrates a significant role for HAS transcripts/proteins in biology of different malignancies. Overexpression of HAS proteins and subsequent overproduction of HA molecules promotes growth and/or metastatic development and tumor progression in fibrosarcoma, prostate, ovarian, colon, breast and endometrial cancer[9-19]. The removal of the HA matrix from a migratory cell membrane inhibits cell movement as has been demonstrated by Banerjee et al. and Evanko et al. [20,21]. Genetic manipulation of the HASs appears to be essential for understanding the role of HA in malignant tumor phenotypes and their basic cellular behaviors. For example, the expression of HAS1 transcripts restored metastatic potential of mouse mammary carcinoma mutants while HAS2 improved the anchorage-independent growth and tumorigenicity of human fibrosarcoma cells, and overexpression of HAS3 promoted the growth of prostate cancer cells[9,22,23]. It appears that the HAS isoenzymes are implicated in different stages in tumor progression. However, the exact relationship between the expression of HASs and the malignant phenotype remains to be determined in clinical samples.

In mouse mammary carcinoma cells, transfection with HAS1 transcripts resulted in the formation of increased metastases as compared to controls[9]. Our work was the first to show an influence of HAS1 expression on survival in human cancer. We find that HAS1 to be abnormally expressed in malignant B cells as described in this thesis[24-26]. Screening of ovarian cancer patients for the expression of HAS1 protein demonstrated that microvessel density was higher in the HAS1 positive group of patients as compared to the HAS1 negative group[17]. However, the microvessel density did not differ in relation to the expression of other HASs in ovarian cancer patients. Additionally, overall survival time was longer in the HAS1 negative group of patients than in the HAS1 positive group. The authors suggest that the HAS1 is an independent predictor of ovarian cancer patient survival and expression of this protein may be associated with disease progression through angiogenesis[17]. Also, association between progression of endometrial cancer and HAS1 expression has been reported[27]. In patients with endometrial cancer, the expression of HAS1 was related to the depth of myometrial invasion, histological grade, and lymph-vascular space involvement. Serum levels of HA were increased as well with depth of myometrial invasion, histological grade, and lymph-vascular space involvement. Furthermore, it has been documented that increased levels of HAS1 transcripts measured by real-time RT-PCR correlate with poor prognosis in human colon cancer[19].

The HAS2 isoenzyme, which is involved in embryonic and cardiac cushion morphogenesis and subsequent development through cell migration and invasion, appears to facilitate abnormal cell proliferation and the activation of cell signaling cascades that stimulate angiogenesis and may promote tumor progression[28,29]. Udabage et al. confirmed the central role of HAS2 in the initiation and progression of breast cancer[14]. Additionally, Mishada et al. suggest a crucial role of HAS2 in osteosarcoma cell proliferation, motility, and invasion[30]. Furthermore, upregulation of HAS2 and HAS3 and subsequent production of HA by these proteins has been shown to promote metastasis in colon canrcinoma[31,32]. Moreover, Itano N. et al. in their study demonstrated that both HAS1 and HAS2 expression were elevated in the highly malignant cells transformed with v-*src* and/or v-*fos*, while expression of HAS3 transcripts were increased in the v-H-*ras* transformed 3Y1 cells which are moderate malignant cells[33]. This study suggests that the regulated expression of the HASs is required for malignant transformation and tumor progression.

HASs, through the synthesis of HA, could facilitate tumor progression through reorganization of the cytoskeleton, including lamellipodial formation, which is a prerequisite for cell spreading and metastatic development. It is also important to note here the significance of focal adhesion formation and cell spreading in metastatic development. Recently Kultti et al. reported maintenance of the microvillous-like cell surface protrusions and accumulation of HA molecules in these protrusions[34]. The authors suggest that the HA accumulated in microvillous-like protrusions can act like as an extracellular cytoskeleton. Additionally, microvillous-like protrusions may facilitate drug-resistance of malignant cells and subsequently disease progression since multi-drug resistance proteins are localized in microvilli formation[35].

All above described processes are accomplished by complex signaling pathways. I do not intend to describe these signaling pathways in detail since they are beyond the scope of the work presented here. However, some examples of these signaling pathways are as follows: overexpression of HAS2 genes in fibroblasts appeared sufficient to induce activation of the HA specific cell surface receptor CD44[36]. Nevertheless, HA produced by overexpressed HAS2 could interact not only with CD44 but with a cell surface receptor for HA mediated motility (RHAMM). HA/CD44 and/or RHAMM interactions have been shown to trigger the activation of the MAPK and PI3 kinase pathways, resulting in active cytoskeleton rearrangement and lamellipodia formation[36,37]. Moreover, overexpression of HAS2 enhances the anchorage-independent growth and tumorogenicity of human fibrosarcoma cells[22,31]. Hall et al. showed that src regulates actin cytoskeleton by a pathway that is triggered by interactions between extracellular

HA and its receptor RHAMM.[38] In this paper, the authors indicate that src-regulated transient phosphorylation of FAK exerts control over cytoskeletal organization.

Interestingly, HA overproduction by cushion cells not only provides a substrate for cardiac cell migration but also influences the transformation of these cells to a motile phenotype, suggesting a significant role in oncogenesis[39]. HA is produced in large quantities by cells undergoing mitosis as well. It facilitates cell rounding and is involved in the post-mitotic separation of daughter cells[40,41]. The activation of HAS isoenzymes appears to be essential for these events. The expression of antisense HAS2 and/or HAS3 in the aggressive prostate adenocarcinoma PC3M-LN4 cell line inhibited tumor growth[23]. This finding suggests that overproduction of HA is required for tumor progression, and that elevated production of HA by prostate stroma and cancer cells is a negative prognostic factor[23]. Overexpression of HAS2 and HAS3 promotes anchorage-independent growth and tumorigenicity in immunocompromised mice[22,23,42]. Compared to HAS1 and HAS3, the HAS2 gene is readily regulated in response to mechanical injury in human peritoneal mesothelial cells *in vitro* and in dermal fibroblasts and osteoblasts in response to glucocorticoids[43-45]. In addition, HAS3 expression has been correlated with increased blood vessel formation[23]. In contrast, Simson et al. showed that decreased vessel formation occurs in tumors as a result of HAS2 overexpression[13]. These divergent outcomes may reflect the synergistic activation of hyaluronidase (HAase) in response to the overexpression of HAS enzymes.

Overexpression of HAS1 or HAS2, both of which appear to synthesize high molecular weight HA may activate hyaluronidase, which degrades HA molecules and which is upregulated or downregulated during the progression of human cancer[28]. Shorter forms of HA resulting from HA degradation have been implicated in angiogenesis[28,29,46].

In conclusion, HAS1 and/or HAS3 gene regulation most likely requires significant changes in the cell or tissue in response to external or internal stimuli. Notwithstanding, little is known about definitive functions of each of the HAS proteins in various types of cancers.

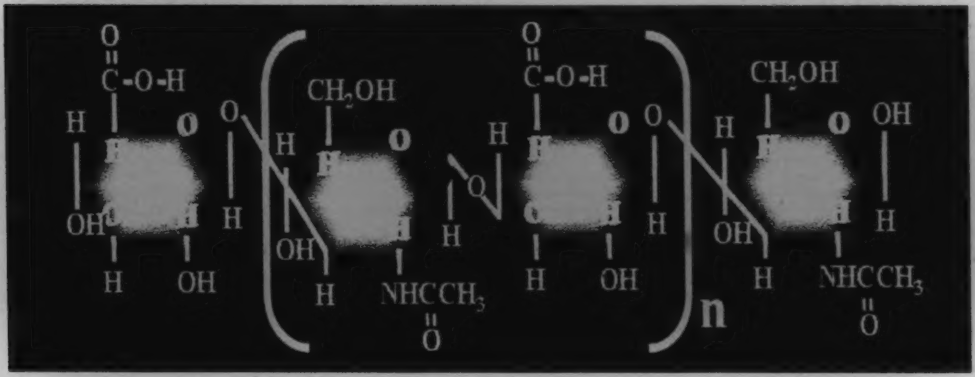
I. 1. 3. Hyaluronan

A number of authors have suggested that the ECM plays a significant role in tumor growth, progression, and metastatic development[47]. Tumor invasion and metastatic development are mediated through the breakdown of the ECM. Disruption of the ECM promotes abnormal inter- and/or intra- cellular signaling leading to abnormal cell proliferation, cell growth, cytoskeleton reorganization, and alteration of other cell functions[47]. HA is involved in many biological processes including malignant cell migration[48,49]. Disruption of the ECM occurs in the case of sustained inflammation; as a result, constitutive activation of stromal fibroblasts leads to upregulation of enzymes such as metalloproteinases (MMPs), ultimately promoting the overproduction of growth factors. These factors endorse aberrant signaling and consequently induce elevated epithelial cell proliferation and promote other abnormalities[47,50]. In addition, disruption of the ECM is a hallmark of cancer and this process, in particular, plays a significant role in tumor progression.

The structural integrity of extracellular and pericellular matrices, which are composed of glycoproteins, proteoglycans (PG), cytokines and growth factors, are retained through the interaction between the negatively charged polysaccharide, HA, and link proteins and/or PG. HA is composed of repeating disaccharide units of D-glucuronic acid and N-acetylglucosamine (Figure 1-2). HA belongs to the family of glycosaminoglycans including heparin sulphate and chondrotin sulphate which are synthesized and assembled in the rough endoplasmic reticulum and Golgi apparatus. However, unlike heparin sulphate and chondrotin sulphate HA molecules are synthesized and assembled at the plasma membrane and are translocated into the ECM[1,4].

HA facilitates its functions through interactions with the HA receptors RHAMM and CD44, as well as with other proteins, including hyaladherins and PG such as aggrecan, versican, neurocan, brevican, which serve as linker proteins[51,52]. HA-PG interactions have significant structural importance in retaining of the ECM and thus facilitating tissue homeostasis[53,54]. The many functions of HA are also regulated through its interactions with the HA binding proteins, the "hyaladherins" such as Hyaluronan binding protein 1 (HABP1). Majumdar and colleagues suggest that HABP1 is a cytoplasmic substrate for MAP kinase, and HABP1 translocation into the nucleus is accomplished by MAPK itself[55]. However, this study also suggests that activation of ERK is a requirement for the translocation of HABP1 into the nucleus. Sequence analysis revealed that this protein is identical to P32 which was co-purified with alternative splicing factor SF2[56,57]. This finding suggests the potential role of HABP1 in splicing machinery, an intriguing finding in light of the results reported in this thesis.

Figure 1-2. Chemical structure of HA



Chemical structure of the HA disaccharide "building block" consisting of N-acetyl-D-glucosamine and glucuronic acid.

Recently Spicer et al. reported the existence of the HA binding link protein gene family (HAPLN)[58]. The authors suggest that the functions of these proteins are analogous to those of cartilage link proteins, but the exact function of this protein family has yet to be elucidated. The data accumulated in the literature clearly suggest the multifunctional role and significance of HA molecules in the biology of vertebrates and humans. Abundant evidence exists to show that upregulation and/or downregulation of HA production can lead to abnormal cell behaviour.

I. 1. 4. Hyaluronan in cancer

Despite its simple chemical composition (see Figure 1-2) HA has been linked with many biological processes including space filling, lubrication, embryogenesis, cell adhesion and motility, cell growth and differentiation and angiogenesis[20,28,39,59-62]. One of the important characteristics of HA is its ability to promote the development and progression of different types of cancer. The association of HA production and tumorigenesis was reported as early as 1989[63].

HA also plays a significant role in maintaining the luminal capillary, arteriole and venule glycocalyces as a barrier[64]. The glycocalyces, in turn, prevent penetration of large macromolecules through the membranes[64]. The glycocalyx, which is a very complex structure, is a dynamic component of endothelial cell function. Dysregulation of endothelial cell matrix formation may promote abnormal angiogenesis, potentially leading to tumor progression. Dramatically increased HA-rich matrix formation has been observed around proliferating and migrating cells during morphogenesis, regeneration and healing. High amounts of HA molecules are synthesized 1) prior to mesenchymal cell differentiation and throughout embryonic development, where the condensation and differentiation of the mesenchymal cells are accompanied by the spatial distribution of HA in the different regions of the limb bud[65-67]; 2) during brain development around proliferating and migrating neuronal cells[68]; and 3) during formation of heart valves when cushion cells migrate from the endocardium to the myocardium[39].

HA creates hydrated pathways, thus facilitating channels for free movement of the cells in this microenvironment[69]. HA molecules are conducive to cell proliferation and migration, preventing differentiation of cells until a sufficient number and appropriate positioning of cells is established, which is essential for the formation of tissues and/or organs[70]. In addition, the formation of hydrated pathways by HA molecules is closely associated with the surface of different types of cells, and these associations promote cell adhesion, an important process in tumor progression[69,71].

An important characteristic of HA is its ability to stimulate malignant cell migration[72-76]. Histological studies of various tumors, using an HA binding protein as a probe, revealed HA as a central component of the stroma which surrounds and supports the tumor[77]. A high level of stromal HA is a strong predictor for the survival of patients with breast and ovarian cancer[77,78]. In addition, overproduction of HA by malignant cells themselves has been detected in patients with multiple myeloma, breast, colon carcinomas, stomach, bladder, non-small-cell lung adenocarcinomas, prostate and ovarian cancers[77-83].

Dahl et al. demonstrated that abnormally high or very low levels of HA in the serum of patients with multiple myeloma (MM) correlated with dramatically reduced median survival of these patients[83]. McBride et al. showed that HA matrices around adherent fibrosarcoma cells act as protective barriers and prevent lysis by cytolytic lymphocytes which are specific to the antigens expressed by the malignant cells[84]. Hyaluronidase treatment of fibrosarcoma cells resulted in the disruption of HA matrix around the fibrosarcoma cell plasma membrane, and also enhanced the cytotoxic action of cytolytic lymphocytes against them[84].

Considering all above-mentioned functions of HA, it seems likely that the impact of HA on malignant growth is multi-factorial. HA molecules present in solid tumors could support the integrity of the tumor tissue and prevent malignant cell differentiation. Alternatively, HA could facilitate detachment of malignant cells from the tumor mass and therefore promote spread of the malignant cells. Furthermore, partial degradation of HA molecules promotes angiogenesis, a vital requirement for tumor growth[29,46]. The precise mechanisms that mediate HA-dependent malignant cell migration remain unclear. HA may contribute to malignant cell migration by increasing tissue hydration and providing a supportive environment for malignant cell migration similar to embryonic cell movement. Thus, controlling HA production, which is produced by the malignant cells, may modulate migration of malignant cells and may contribute to tumor dormancy by inhibiting malignant spread.

Alternatively, HA may exert an anti-tumor effect when administered exogenously. In a xenotransplant model of human breast cancer, intratumoral injection of HA was shown to inhibit tumor growth and promote tumor regression[85]. HA reverses myeloid differentiation blockage in acute myeloid leukemia cells[86]. For astrocytoma cells, HA was found to decrease invasion[87]. Finally HA oligomers inhibit anchorage independent growth of tumor cells[88]. Thus, these observations suggest the therapeutic use of HA to inhibit tumor growth and spread.

I. 1. 5. Intracellular HA

In addition to extracellular HA molecules which are extruded into the extracellular compartment, an intracellular form of HA has been detected in the cytoplasm, nucleus and nucleoli peripheries of various tissues such as the brain, liver, arteries, cumulus cells and oocytes[40,89-96]. Evanko et al. showed localization of intracellular HA during mitosis at the metaphase plate[40]. Furthermore, these molecules were detected around chromosomes during their rearrangement and separation in anaphase. In addition, accumulation of HA in fibroblasts increased motility of these cells[97,98]. Recently Evanko et al. demonstrated association of intracellular HA with intracellular RHAMM. Furthermore, a central role of an intracellular HA in inflammatory processes has been precisely reported in a review by Hascall et al.[62]. In addition to extracellular HA, an intracellular HA has been detected throughout cell division[40,62,90]. Abnormal expression of the HA molecules either inside or outside the cell during mitosis may promote abnormal cell proliferation and as a result, tumor progression.

In the literature it is suggested and very elegantly documented by Tammi that accumulation of intracellular HA in the cells occurs through the endocytic route with involvement of CD44[99]. However, this model cannot accomodate accumulation of intracellular HA by malignant cells that lack expression of CD44. Moreover, Eggli and Graber detected HA molecules within caveolae of endothelial and smooth muscle cells[89]. Thus, the source of intracellular HA and its function remain unclear.

I. 1. 6. HA receptors RHAMM and CD44

Two HA specific receptors, RHAMM and CD44, have been identified thus far in humans. The coiled-coil protein RHAMM is distributed in different compartments of the cell, including cell surface, nucleus and cytoplasm[49,100-106]. Overexpression of RHAMM promotes malignant cell migration and leads to metastatic development and tumor growth[107,108]. RHAMM is implicated in the Ras signaling pathway which is altered in all types of cancer[109]. Two splice variants of RHAMM have been identified: RHAMM^{-exon4} and RHAMM$^{-exon13}$, both of which are overexpressed in MM and other B lymphocyte (such as B-CLL and lymphoma) as demonstrated by Crainie et al.[110].

RHAMM is involved in cell signaling and migration via interactions with HA, microtubules, and calmodulin[38,90,97,98,100,111,112]. Assmann et al., first demonstrated that intracellular RHAMM interacts with microtubules and microfilaments and may mediate cell locomotion through these interactions[100]. The same study showed that the RHAMM protein has the capacity to bind to the regulatory protein calmodulin in a Ca^{2+} dependent manner[100]. RHAMM-calmodulin interactions may be controlled by activation or deactivation of HAS proteins, since it is known that increased production of HA, resulting from activation of HASs, increases concentration of intracellular Ca^{2+} [113]. It seems that RHAMM and HASs act in concert to promote tumor progression through cytoskeletal rearrangement. Thus, the inhibition of HAS proteins may be an effective target to downregulate RHAMM mediated malignant cell migration.

In addition, RHAMM has been identified as a centrosomal protein that mediates the stability of the mitotic spindle[114]. Maxwell et al. in their study demonstrated that disruption of RHAMM dyenin-dyenactin interaction leads to multiple spindle formation, which in turn promotes missegragation of the chromosomes and latter on facilitates aberrant mitosis[114]. Thus, the domains of RHAMM that bind HA are also responsible for the centrosomal localization of RHAMM. These findings suggest that the c-terminus of RHAMM mediates the multiple functions of this protein. Furthermore, in H-*ras*-transformed fibrosarcoma cells inhibition of RHAMM receptor results in cell cycle arrest in the G2/M phase through suppression of cdc2/cyclin B1 expression[111]. Thus, RHAMM appears to be a key regulator of cell growth and plays a significant role in metastatic development.

CD44 appears to be a major binding site of HA for many cell types. Ten splice variants of CD44 have been identified thus far. The interaction of HA with CD44 has been implicated in cell proliferation, migration and angiogenesis[115-119]. For example, CD44 clustering on the surface of mammary carcinoma and melanoma cells leads to docking of gelatinase B, an enzyme which promotes tumor invasion and angiogenesis[120,121]. CD44 also mediates a variety of intracellular signaling cascades and interacts with cytoskeletal proteins, thus promoting specific signaling pathways that are essential for the normal functioning of cells. However, aberrant activation of these signaling pathways can lead to malignant behavior.

HA activated CD44 directly interacts with p185HER2 and c-src, and regulates components of the cytoskeleton such as actin filaments and microtubules[60,122,123]. These interactions mediate cytoskeleton-regulated cell migration. The cytoplasmic domain of CD44v10 is a substrate for PKC. The phosphorylation of CD44v10 by PKC enhances interaction of CD44v10 with cytoskeletal proteins and as a result promotes rearrangement of the cell cytoskeleton. Moreover, another variant of CD44, CD44v3, is predominantly detected in highly malignant breast carcinoma tissue samples[124-126]. CD44v3 serves as a substrate for Rho-A activated ROK which phosphorylates the cytoplamic domain of CD44v3[127]. Next, CD44v3 interacts with the cytoskeletal protein ankyrin, promoting malignant cell migration in patients with breast tumors. Furthermore, ROK is involved in cross-talk between Ras and Rho signaling pathways, thus leading to cellular transformation which favors malignant cell proliferation and motility. Additionally, CD44-HA interaction promotes intracellular Ca^{2+} mobilization which activates various signaling pathways including Ca^{2+} dependent IP_3 and Ca^{2+}/calmodulin dependent signaling pathways. Ca^{2+}/calmodulin, in concert with Rho-A and Rac1, regulates cytoskeletal function through phosphorylation of many important cytoskeleton regulating proteins such as the myosin light chain. The activated myosin light chain has been shown to promote endothelial cell migration and thus play an important role during angiogenesis[127].

However there have been no studies to show that expression of CD44 is absolutely necessary for HA mediated metastatic development. HAS proteins themselves in their structure include HA binding sites (see Figure 1-1) and thus can retain an HA matrix around the cells not expressing CD44 receptors. This also suggests that CD44 mediated tumor progression is more complex and extends beyond CD44-HA interactions. For lymphocytes, CD44 is usually abundantly expressed as compared to RHAMM, which is usually expressed at relatively low density.

Despite intensive studies conducted thus far, the complete function of HA molecules in eukaryotes is not fully understood. Nevertheless, HA appears to be vitally important since many inherited diseases detected thus far are accompanied by aberrant HA production and turnover of this polysaccharide. As summarized above, HA-cell interactions have a major impact on normal and malignant cell behavior. Taken together, the body of work describing the impact of HA and HASs in cancer and other diseases makes it abundantly clear that the HA and the HAS isozymes should be important targets for novel strategies to eradicate and/or control malignant growth and progression.

I. 2. 1. Alternative splicing

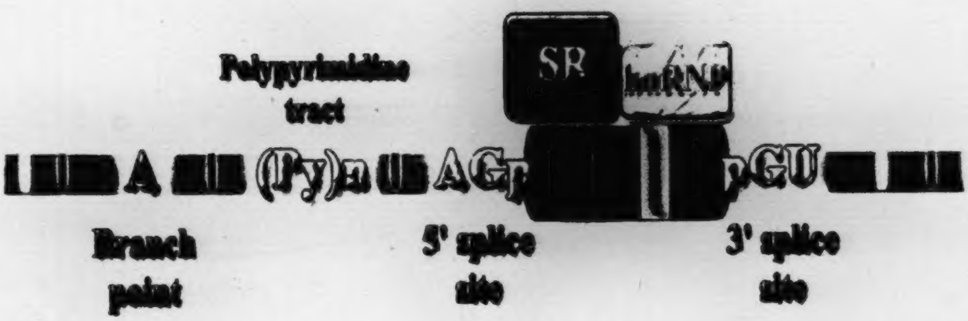
The essential dogma of molecular biology states that DNA is transcribed into messenger RNA (mRNA) and that these mRNA transcripts are translated into proteins in the cytosol of the cell. There are fundamental differences between prokaryotic and eukaryotic mRNAs. A prokaryotic mRNA does not need to be processed or transported to the place of translation, as transcription takes place in the cytosol. The translation in prokaryotes begins on the ribosomes immediately after transcription starts. In eukaryotes transcription occurs in the nucleus. The primary product of transcription is pre-mRNA, which undergoes complex processing before leaving the nucleus.

Pre-mRNA, the long precursor of eukaryotic mRNA, contains coding regions known as exons interrupted by much longer non-coding regions of a gene called introns. In pre-mRNA molecules, before leaving the nucleus, introns are removed and exons are joined by the precise process called splicing. This process is catalyzed co-transcriptionally by the spliceosome which is a large RNA-protein complex composed of five small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5, and U6. Each snRNP is composed of uridine rich RNA and protein. Splicing of a given gene requires activation of more than 100 proteins, including splicing factors and the 5 snRNPs listed above[128].

Splicing starts with the recognition of splice sites by snRNPs on exon-intron boundaries of pre-mRNA. Alterations in splice site choice have different effects on mature mRNA and consequently on the protein products of a gene. Aberrant mRNA transcripts are either unstable or encode “defective” protein isoforms that differ from each other in peptide sequence. Consequently, the chemical and biological activity of these protein isoforms will be different from their wild type counterparts[129,130]. Nevertheless, the splicing process is necessary not only to remove introns from a pre-mRNA and to generate correct transcripts for protein production, but splicing also is required to generate functionally diverse protein isoforms in a regulated manner, thus maximizing the information encoded in the human genome. In eukaryotic organisms some genes exhibit a few splicing patterns, while others can generate thousands of splice variant transcripts[131,132]. At least 60% of the genes in the human genome undergo splicing[133,134].

Accuracy of splicing is maintained by classical splice elements, including the 5' splice site known as the splicing donor, and 3' splice site termed the splicing acceptor (Figure 1-3)[130,135]. These conserved consensus sequences of 5' and 3' splice sites are located on the exon-intron boundaries of a pre-mRNA[130]. The 5' splice site defines the exon-intron boundary at the 5'end of any given intron. A dinucleotide GU is conserved within the less conserved consensus sequence of the 5' splice site[130,136]. The 3'end of any given intron consists of three conserved sequence elements including a splicing branch point (BP), polypyrimidine tract (PPT) of splicing, and the 3'splice site which has a terminal AG dinucleotide at the 3' end of the given intron (Figure 1-3)[130,136,137]. In addition to these classical splicing elements, the complex mechanism of splicing requires other elements to modulate splicing in cell-type specific manner. These additional elements are *cis*-splicing elements, comprised of the sequence motifs, distributed on the exons and introns of a gene (Figure 1-3)[138,139]. Some of these elements enhance splicing, while others suppress splicing; they are known as splicing enhancers and suppressors

Figure 1-3. Alternative splicing elements



This figure describes locations of classical and *cis*-splicing elements. The dark blue tube represents the exon, while the light blue tube represents the introns. Classical splicing elements are: AGp—5'splice site, pGU—3' splice site, "A"—Branch point of splicing, (Py)n—polypyrimidine tract of splicing. Red lines on the exon and introns are exonic and intronic enhancers (ESE, ISE), which attract SR (serin/arginine rich) proteins. Light blue lines on the exon and introns are exonic and intronic silencers (ESS, ISS). These sequence motifs usually attract hnRNPs (heterogeneous nuclear ribonucleoprotein).

accordingly[136,137]. There are two types of enhancers and suppressors: exonic (exonic splicing enhancer/suppressor—ESE/ESS) and intronic (intronic splicing enhancer/suppressor—ISE/ISS).

The most prevalent cis-splicing elements are exonic splicing enhancers (ESE). They are present mostly on exons[137,140,141]. Splicing enhancer sequence motifs attract members of the serine/arginine-rich (SR) protein family of splicing factors (Figure 1-3)[140,142]. These proteins have one or two N-terminal RNA recognition motifs (RRM) which determine binding specificity for pre-mRNAs. The C-terminal domains of SR proteins are enriched with serine and arginine residues and are involved in protein-protein interactions. SR protein binding to pre-mRNA in context with classical splicing elements determines exons within this pre-mRNA and promotes further accumulation of the proteins involved in the splicing process[140,143-145]. The most studied SR proteins are SF2/ASF, SRp20, SRp40, SRp55, and SC35. The functions of these SR proteins and additional members of the SR family of proteins are summarized in the reviews by Matlin et al. and Bourgeois et al.[146,147].

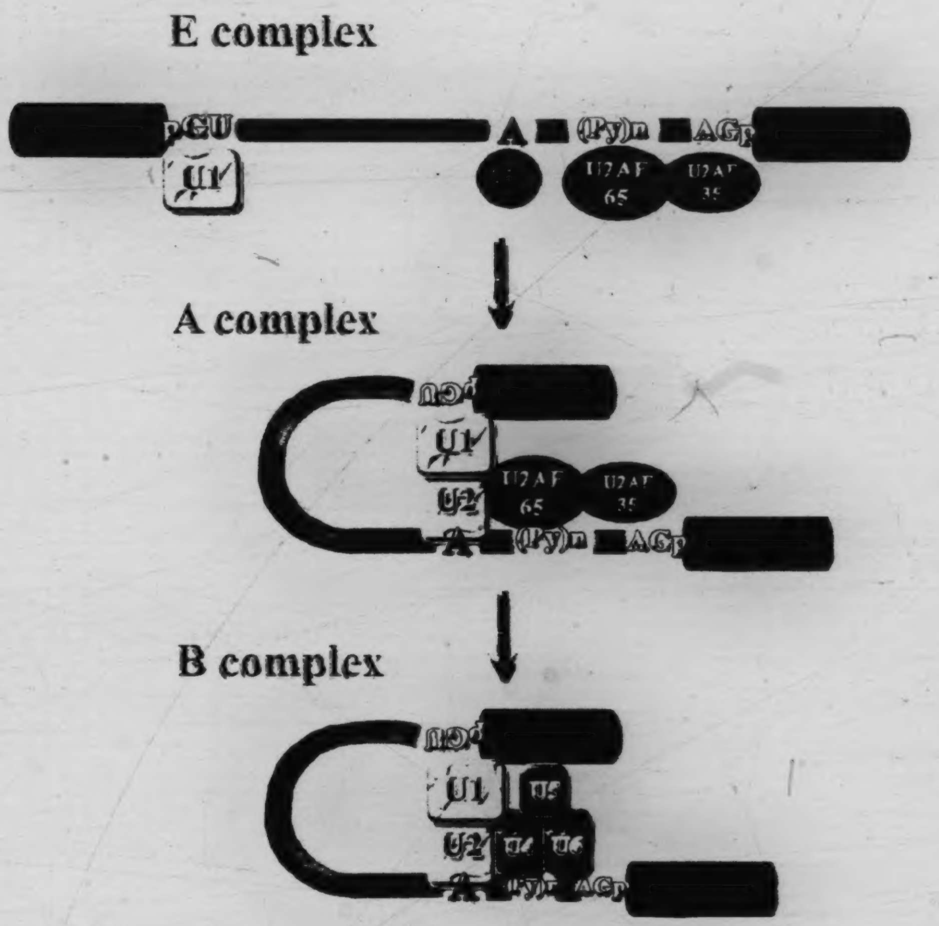
The most described splicing silencers are located on introns (ISS-intronic splicing silencer), however a number of exonic splicing silencers (ESE) have also been described [130,148-150]. ESS and ISS attract family members of the heterogeneous nuclear ribonucleoproteins (hnRNPs) which are in most cases negative regulators of splicing (Figure 1-3)[147,151,152]. Similar to the SR proteins, hnRNPs have an RNA binding domain, an RRM motif, and auxiliary domains that are involved in protein-protein interactions and play multiple role in pre-mRNA and mRNA metabolism[153,154]. The intronic regulatory sequences are located close to the exon-intron boundaries. However, some of these cis-splicing elements are distributed deep within the introns[130,155]. The most prevalent and studied hnRNPs in a splicing context are hnRNP A, hnRNP I (known as polypyrimidine tract binding protein—PTB), hnRNP A/B, and hnRNP H[156,157]. Details of the actions and the effects of hnRNPs on the splicing process are well summarized in a review by Moore et al.[136].

Thus, previous and recent studies suggest that the splicing decision is not restricted to a static pre-synthesized template. On the contrary, as soon as pre-mRNAs are transcribed from DNA they become "frosted" with different types of splicing factors, co-factors, and proteins involved in the complex process of splicing.

I. 2. 2. The steps of splicing

As mentioned above, pre-mRNA splicing which takes place in the cell nucleus is responsible for the production of mature mRNA from a single gene. In some cases, the splicing process can generate many unique proteins through variations in the splicing of the same mRNA. This phenomenon is called alternative splicing. Physiological functions of alternative splice variant products are usually different from wild type product and in some cases completely different. This explains how human complexity arose from a relatively small number of genes. The splicing process is accomplished through two transesterification steps (Figure 1-4 and 1-5).

Figure 1-4. Spliceosomal assembly — initiation of splicing process

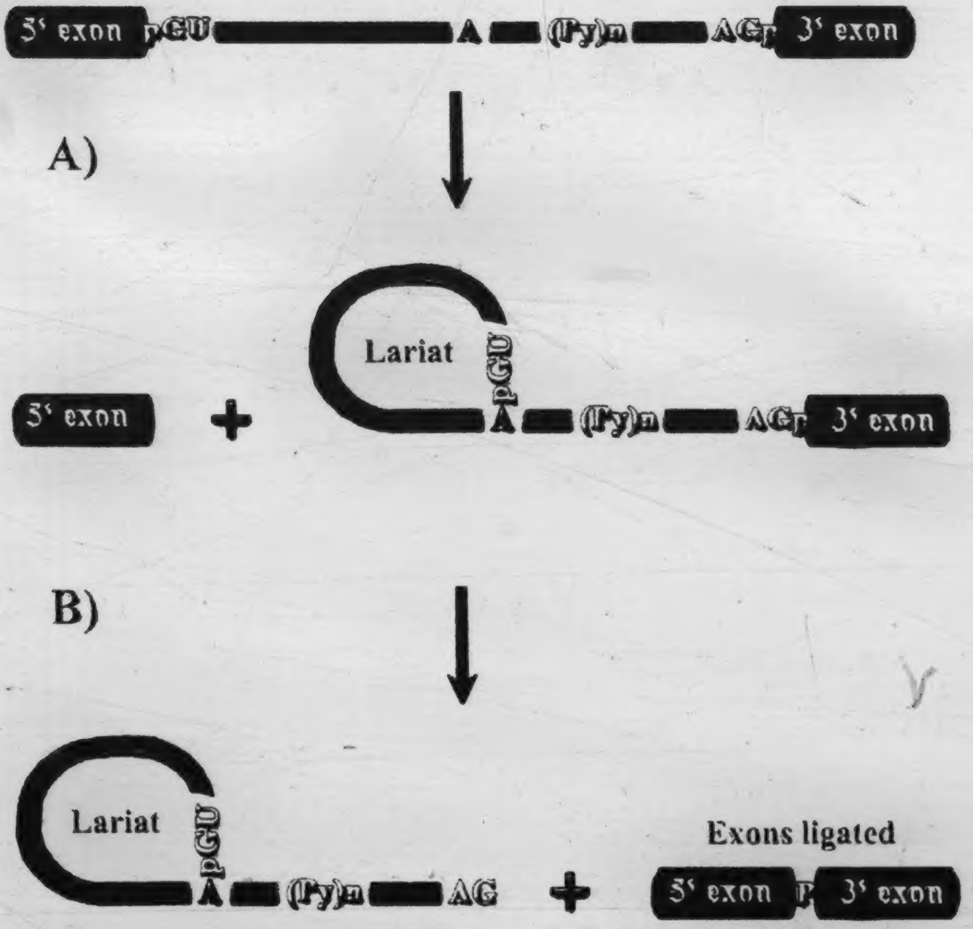


Spliceosomal assembly and formation of complexes E, A, and B are the steps that precede the first transesterification reaction which is catalyzed by the C complex. The C complex is formed as a result of extensive rearrangement of Complex B. U1, 2, 4, 5, and 6 are small nuclear ribonucleoproteins i.e. snRNP that comprise the spliceosome, while SF1 is a branch point binding protein and U2AF is the U 2 auxiliary factor. See text for details. This Figure is adapted from Black et al. [130]

During the transesterification reaction, four snRNPs and additional other proteins bind to pre-mRNAs in a specific order[130,146]. As the first step of transesterification, the snRNPs must recognize the exon–intron boundaries[130,136]. This process involves RNA to RNA base-pairing which is mediated through RNA-protein interactions. The U1 snRNP binds to the 5'splice site of an intron which is subjected to alternative splicing (Figure 1-4 A). U1 forms the complex between the 5'spice site and the U1 containing RNA. Second, 3' splice elements are bound in specific order to the following proteins: SF1 binds to a branch point of splicing and is called branch point binding protein—BBP, the 65 KD subunit of U2AF protein binds to the polypyrimidine tract, and the 35KD subunit of U2AF binds to a 3'splice site (Figure 1-4 A)[130,136,146]. The binding of 65KD and 35 KD subunits of U2AF to the branch point and polypyrimidine tract respectively defines the binding of U2 to the branch point of splicing located in close proximity to the 3' splice site. After binding of all above mentioned proteins to the classical splicing elements, the first spliceosomal complexes, the E complex (early complex) and complex A, are assembled (Figure 1-4 A, B). Complex A is formed between the branch point and U2 with the aid of protein factor U2AF SR protein (Figure 1-4 A, B). After formation of complex A, U2AF is displaced with U4/U5 and U6 to form complex B (Figure 1-4 C)[130,146]. At this point SF1 and U2AF are displaced from the classical splicing elements. Formation of the B complex is followed by a conformational rearrangement occurring in the spliceosomes and pre-mRNA that lead to formation of the C complex. Formation of A, B and E complexes initiates the first transesterification reaction, while C complex promotes catalysis. U4 disassociates from U6 and U6 is placed in position to catalyze the first transesterification reaction (Figure 1-5 A)[130,136,146]. In this reaction the 2'-hydroxyl group of the branch point adenosine residue attacks the phosphate group of the 5' splice site leading to cleavage of the 5'splice site from the adjacent exon. As a result the 5'splice site is ligated to the branch point (Figure 1-5 A). Immediately after this process the second transesterification reaction takes place.

This reaction leads to a cleavage of the 3'splice site from the adjacent exon, with the spliced out intron being excised as a lariat structure (Figure 1-5 B). The second transesterification reaction also leads to the ligation of exons and spliceosomal disassembly from this region (Figure 1-5 B). This reaction involves attack by the 3'hydroxyl group of the detached exon on the phosphate group at the 3'end of the involved intron[130,136,146].

Figure 1-5. Splicing reactions



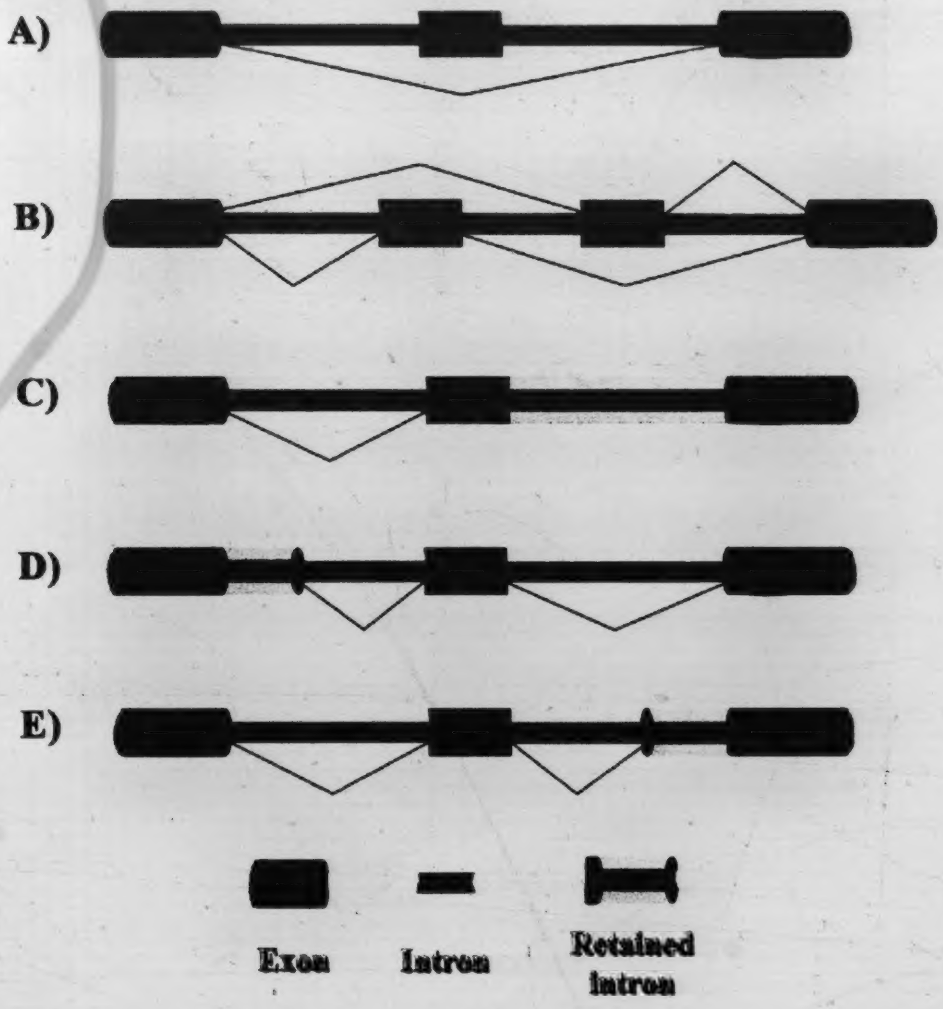
A) The first transesterification reaction results in a detached 5' exon and the 3'exon/intron in a lariat structure; B) The second transesterification reaction liberates the intron lariat and ligates the exons. Details of these reactions are described in the text. This Figure is adapted from Black et al.[130]

I. 2. 3. Modes of alternative splicing

As described above, the proteins responsible for the splicing specifically bind to classical splice sites which are specific sequence motifs on pre-mRNA. It has been demonstrated that the binding of key splicing proteins to splicing sites and consequent spliceosomal assembly are coordinated through the proteins recruted by the cis-splicing elements such as ESE, ISE, ISE, and ISS[155,158]. These cis-elements facilitate correct identification of the exon-intron boundaries of a gene and thus prevent inclusion of pseudo-exons. In addition, proteins accumulated on cis-splicing elements are able to activate cryptic splice sites and repress native sites[151,155,158]. It has been demonstrated that correct splicing can be altered not only by mutations occurring on the classical splice sites, but also by genetic variations that take place within the cis-splicing elements. Furthermore, deep intronic mutations occurring on cis-elements can change splicing[158-162].

Thus far at least 5 different patterns of alternative splicing have been observed: 1) exon skipping i.e. exclusion—this type of splicing is one of the most conserved splicing patterns in humans (Figure 1-6 A); 2) a cassette exon inclusion or exclusion. Most exons in pre-mRNA are constitutive and are always included in the final mRNA transcripts during the splicing process. However, some exons are regulated and can be either included or excluded from the final mRNA transcripts; these exons are called cassette exons. In some cases multiple cassette exons are mutually exclusive and generate mRNA that includes only one exon of a group (Figure 1-6 B); 3) entire intron retention which results in the usage of an intron sequence as an exon (Figure 1-6 C); 4-5) partial

Figure 1-6. Modes of alternative splicing



A) Exon skipping or inclusion. B) Mutually exclusive exons: during splicing only one will be included in the mature mRNA. C) Intron retention. D) Partial retention of the intron as a result of activation/creation of a 5'cryptic splice site. E) Partial retention of the intron as a result of activation/creation of a 3'cryptic splice site. This figure is adapted from Cartegni et al.[158]

retention of an intron at the 5' splice site or 3' splcie site. In both cases mutations occurring on classical 5'- of 3'-splice sites or within cis-elements activate or create 5' splice or 3'splice sites. These genetic variations lead to a partial retention of introns in mature mRNA, usually as an aberrant event in malignant cells (see below). Thus, alternate usage of 5' and/or 3' splice sites cause changes in the size of an exon (Figure 1-6 D, E)[158,163].

In addition to the above listed patterns of alternative splicing, novel transcripts of a gene can be obtained by switching the 5'terminal exon and using alternative promoters or by switching the 3' terminal exon which results truncation of the transcript through alternative splicing with alternative polyadenylation sites. Also, one single pre-mRNA could comprise combinations of the above listed patterns of alternative splicing and produce many different mature mRNA transcripts.

I. 2. 4. Alternative splicing and cancer

The products of alternative splicing patterns that have been described will yield proteins with different physiological functions, or they could act as dominant-negative inhibitors and abrogate the normal function of wild type proteins[164,165]. Aberrant splice events can also transform membrane-bound proteins into soluble proteins[166]. Currently, many of the cancer-related genes identified so far are subject to alternative or aberrant splicing. Furthermore, splice variants of these genes either cause disease, are involved in disease development and progression, or have been used as cancer markers. The most common are CD44, BRCA1/BRAC2, MDM2, the kallikrein family, and the fibroblast growth factor receptor family.

CD44, a multifunctional receptor, has a minimum of 20 splice variants, expression of which have been associated with many different types of cancer. Expression of some CD44 variants appear to be cell type-specific. An extensive body of work describes associations of CD44 variants with cancer. Some CD44 variants are used for tumor imaging, while others are used as an indicator of tumor progression[167-169]. Downregulation of CD44s and CD44v6 variants has been shown to correspond to unfavorable prognosis in prostate cancer while overexpression of CD44v7-9 has been detected in benign prostate tissues[170]. These are several examples showing the role of CD44 variants in different types of cancer[116-119,171,172].

Forty splice variants of MDM2 have been identified in tumors. Many MDM2 variants, which are restricted to tumors, exhibit a transforming function[173]. Expression of MDM2 splice variants is associated with the most aggressive breast cancers[174]. Furthermore, alternative and aberrant splice variants of MDM2 have been detected in ovarian and bladder cancers, glioblastomas and soft-tissue sarcomas[175-178]. Currently little is known about the functions of the proteins encoded by splice variants of MDM2. Interestingly, splice variants of MDM2 are usually detected with the full length transcripts of this gene. Evans et al. in their study demonstrate that at least one splice variant of MDM2, particularly MDM2 B known as ALT1, is able to bind to the full-length of this protein and sequester endogenous MDM2 in the cytoplasm[179]. Evans et al also showed that interaction between MDM2B and full length MDM2 promoted the activation of wild-type p53[179]. The study conducted by Evans et al in context other findings suggest that in some cases accumulation of p53 is a result of MDM2 aberrant splicing and is independent of the mutational status of p53[179-181]. In conclusion, CD44 and MDM2 are the best examples to describe associations between splice variants and cancers.

I. 3. 1. Multiple myeloma statistics and clinical features

MM presently is an incurable disease and accounts 1% of all malignancies, 10% of all hematological cancers, and 19% of deaths from hematological malignancies. An estimated 1900 (850 female and 1050 male) new MM cases and 1300 (620 female and 700 male) deaths of individuals with MM will occur across Canada in 2006 (Canada Cancer Statistics 2006; www.cancer.ca). In Canada this malignancy remains one of the worst cancers with respect to survival rate (death to case ratio is 0.68) as compared to more common cancers such as lung, colorectal, prostate, and breast. The incidence of MM is twice as high in African Americans as in Caucasians and slightly more common in men than in women. Often MM is preceded by a premalignant condition termed monoclonal gammopathy of undetermined significance (MGUS). MGUS occurs in about 3% of individuals over the age of 50.

According to the World Health Organization (WHO) classification, MM belongs to the category of B cell neoplasm and is further subgrouped with a spectrum of disorders termed plasma cell dyscrasias. MM is a disease of B-lymphocytes which mature to malignant plasma cells (PC) as disease develops. Clinically MM shares some similarities with other diseases such as Waldenstrom's macroglobulinemia (WM), plasmacytomas, plasma cell leukemia, and primary amyloidosis.

MM is a heterogeneous disease manifested by the accumulation of monoclonal immunoglobulin (Ig) in the blood of patients, osteolytic bone disease, impaired hematopiesis, hypercalcemia, and renal failure[182]. MM clone is characterized by a unique IgH VDJ gene rearrangement which provides a unique molecular signature for the malignant cells of each patient. The common presenting clinical symptoms of MM are bone pain, recurrent infections, and fatigue[182]. Prevalence of MM increases with age. In a new International Staging System, which is established based on the information collected from 17 institutions worldwide, MM patients have been grouped into 3 distinct stages, Stage I, II and III, based on serum β_2-microglobulin and albumin levels[183]. Stage I includes patients with a serum albumin level of more than 3.5g/dL and a serum β_2-microglobulin of less than 3.5 ug/ml. Stage II includes patients which are neither Stage I or II, and Stage III is comprised of patients having serum β_2-microglobulin levels more than 5.5 ug/ml. The estimated median survival of patients included in Stage I was 62 months, for Stage II 44 months, and Stage III 29 months.[183] Patients with asymptomatic disease are not included in this staging system. The patients with asymptomatic disease are evaluated as having indolent or smoldering MM and are left without treatment until the disease progresses to more aggressive stages.

I. 3. 2. Abnormalities detected in MM patients

The etiology of MM in humans is unknown. Although there have been attempts to establish connections between the MM and certain infectious diseases through epidemiological studies, no definitive conclusions have been possible. Although some familial cases of MM have been observed, the low incidence of familial MM has hindered understanding of the genetic predispositions for this disease.

Extensive cytogenetic studies suggest that all cases of MM are characterized by chromosomal abnormalities including numerical and structural abnormalities.[184-198] Recently, based on chromosomal abnormalities, MM patients are divided into two groups: hyperdiploid and non-hyperdiploid[187,190,199-201]. Hyperdiploid MM cases, which account for nearly half the MM population, are characterized by multiple trisomies which involve chromosomes 3,5,7,9,11,15,19, and 21[184,197,202-205]. These patients have a chromosome count close to 53. In addition to trisomies, monosomies of chromosomes 13 and 14 are relatively frequent in MM and appear to have a particularly poor prognosis[184,197,206].

As mentioned above, MM patients are characterized by structural chromosomal abnormalities which include primary and secondary chromosomal translocations. Among these translocations the most important are the recurrent IgH translocations on the 14q32 locus. These primary translocations include t(11;14) and t(6;14) involving cyclin D 1 and cyclin D3 genes respectively, t(4;14) involving the upregulation of FGFR-3 and MMSET, and t(14;16) and t(14;20) involving up-regulation of C-MAF and MAF-B.

These translocations are present in 45% of the patient population and this number is slightly increased in the patient population with advanced disease. The other 20% of patients with MM exhibit translocations involving other chromosomal partners. These non-recurrent translocations are found in 1% or less of MM patients[207,208]. The clinical and pathological implications, including prognosis, of these translocations is reported in a review by Higgins and Fonseca[209]. Of note, the prevalence of IgH translocations in MGUS patients appears to be as high as it is in MM patients, but this observation suffers from technical limitations of the methods used. Currently, the question about translocation partners and their contribution to biology of the disease and clinical behavior is under investigation. Of note, it is also very important to study the genetic abnormalities in patients without chromosomal translocations.

Other secondary genetic variations that have been detected in MM patients include translocations involving c-MYC, mutations in N-RAS and K-RAS, and abnormalities in p53[210-214]. In addition to genetic abnormalities there are a number of growth factors and signaling molecules involved in the pathogenesis of MM. These molecules include IL-6, tumor necrosis factor, vascular endothelial growth factor, and insulin-like growth factor. However MM is characterized by extensive inter- and intraclonal heterogeneity of chromosomal abnormalities. There are as yet no common phenotypic or genotypic markers identified that would distinguish MGUS from early stages of MM. Thus, it remains impossible to predict which MGUS patients will progress to MM and when.

I. 3. 3. Waldonstrom's macroglobulinemia: clinical features and abnormalities

According to WHO classification, WM is considered to be a lymphoplasmacytic lymphoma. However, WM is a distinct clinicopathological entity characterized by the presence of a monoclonal protein, IgM, in the serum and infiltration of the bone marrow (BM) by small lymphocytes, plasmacytoid cells, and mature plasma cells. WM is an uncommon disease originally described in 1944 by Dr. Jan Waldenstrom. He reported two patients with anemia, lymphadenopathy and hypergammaglobulinemia[215]. WM accounts for 2% of hematological malignancies and has a median survival range from 5 to 10 years[216]. As Dimopoulos et al. suggest, discrepancies in median survival range of WM patients reported to date may reflect the inclusion criteria used. Furthermore patient survival at different centres has been correlated with different clinical and laboratory variables[217]. Unlike MM, the incidence of WM is higher among Caucasians, and only 5% of all patients are of African descent. WM is disease of older adults, with a slight predominance of male over female[218]. Recent epidemiological studies suggested that 20% of 181 WM patients had a first degree relative with WM or with another B cell disorder[219,220]. The etiology of the disease is unknown.

WM is highly heterogeneous with respect to presenting clinical features. Small numbers of patients are asymptomatic and the high levels of IgM characteristics of this disease have been detected in some of these patients during unrelated clinical investigations. However, the most common presenting clinical features of symptomatic WM are weakness, anorexia, lymphadenopathy, organomegaly, and weight loss, in addition to

IgM paraproteinemia. Symptoms due to elevated IgM include hyperviscosity, cryoglobulinemia, cold agglutinin, neuropathy, and amyloidosis. Amyloidosis is a rare complication of WM. The symptoms, which are due to peripheral neuropathy and Raynaud's phenomenon, are manifested when the disease progresses. Standards for diagnosis and monitoring of WM were recently established at the Second International Workshop on Waldenstrom's Macroglobulinemia[221,222].

Known chromosomal abnormalities related to WM are limited. Unlike other B cell neoplasms but like MM, WM lacks a karyotypic signature because of the low yield of abnormal metaphases. Several cytogenetic and genetic studies have been performed on small cohorts of WM patients[223-226]. These studies demonstrated that WM patients, unlike those with many other B cell malignancies, do not harbor translocations involving the IgH locus on chromosome 14q32[226,227]. Schop et al reported that the aneuploidy detected in MM is absent from WM patients[228]. However, the true incidence of aneuploidy in WM patients is unknown. The most common cytogenetic abnormality, detected in 40%-90% of WM cases, is a deletion of the long arm of chromosome 6[225,226,229]. However, this deletion is not limited to WM patients. Recently, gene expression profiling analysis conducted on small cohorts of WM patients (73 patients) showed upregulation of a small set of genes in CD19$^+$ bone marrow cells obtained from these patients. Among these genes, up-regulation of IL-6 was the most significant[230]. However, the biological function and clinical significance of this gene product in WM has yet to be determined. Of note, IL-6 is a major growth factor which is up-regulated in MM. IL-6 stimulates expression and secretion of vascular endothelial growth factor

(VEGF) in plasma cells from patients with MM[231]. Thus, specific genetic markers which reflect biology of WM and which can be used in the routine diagnostic setting still remain elusive. Phenotypic analysis of WM cells demonstrated that the majority of WM malignant cells express the pan-B cell markers CD19, CD20, CD22, CD79, and FM7 while expression of CD10 and CD23 has been rarely seen[232,233]. Also, monoclonal WM cells express sIgM and sIgD[234]. Based on these results it has been suggested that WM B cells may originate from IgM$^+$ or IgM$^+$IgD$^+$ memory B cells[234,235].

I. 3. 4. Hypothesis

Although extensive studies suggest the influence of multiple genetic variations in the progression of MM, these abnormalities have been detected in malignant plasma cells which are quiescent, having low proliferative properties. Thus, it is questionable whether these cells have the ability to initiate and maintain progression of this disease. Consequently, it is logical to think that abnormalities found in the quiescent malignant MM plasma cells may reflect disease activity and not the generative biology of the disease. This may explain why the outcome for MM patients is highly heterogeneous. Since the identity of progenitor MM cells remains controversial, the true primary molecular abnormalities which direct MM precursor clones towards more aggressive disease are unknown. For the same reason, identification of potential predisposing or early stage markers of MM has been hindered. Thus, stratification of patients for treatment is not clinically feasible. Although over the last 10 years the information about WM disease has been increased, the primary genetic and molecular pathways that trace the biology of this disease also remain ambiguous. As a result, even to distinguish WM from other lymphpoproliferative diseases remains a challenge. Consequently, stratification of WM patients for any specific therapy is still difficult or impossible.

Thus, the "cure" of these diseases requires identification of molecular aberrations that lead MM and WM precursor clones to give rise to malignant clones and direct them towards transformation. Elucidation of fundamental genetic abnormalities will help to identify the targets which play a critical role in pathophysiology and outcome of MM and WM. Next, identification of those genetic changes associated with either a good or a poor prognosis will help to stratify patients according to risk and adapt treatment to the expected outcome.

The work reported in this thesis investigated the role of HAS in genesis of MM and WM. **The overall hypothesis of this thesis is that expression of HASs and aberrant splicing of HAS1 are one of the primary genetic abnormalities that contribute to the generation of aggressive MM and WM clones.**

I. 3. 5. References

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Chapter 2: Intronic splicing of hyaluronan synthase 1 (HAS1): a biologically relevant indicator of poor outcome in multiple myeloma

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S.A..independently conceived the idea, designed and performed the research, data analysis and interpretation of the results wrote the manuscript; T.R. conducted statistical analysis, assisted in writing paper; M.C. started the projected and identified HAS1Va splice variant; M.J.M. provided patient samples; A.R.B. served as director of hematology clinic at the Cross Cancer Institute, provided clinical contributions and intellectual contributions to the manuscript; L.M.P. supervised and designed research and assisted in data interpretation.

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Chapter 2. 1. Introduction

Multiple Myeloma (MM) is an incurable malignancy of bone marrow (BM) plasma cells (PC) with a median survival rate of 3-4 years post diagnosis. Molecular studies conducted by us and others have identified CD19$^+$, late stage B cells in the peripheral blood (PB) of patients with MM[1-4]. They are characterized by a clonotypic IgH VDJ rearrangement identical to that of malignant PCs in the BM[1,3,4]. MM B cells are drug-resistant and express hyaluronan (HA) specific receptors RHAMM (Receptor for HA mediated Motility) and CD44, that are necessary for MM cell motility or adhesion[5-9].

Previously, we have shown that the motility of circulating MM B cells is mediated by the extracellular matrix molecule HA[6]. High or low levels of HA in the serum of patients with MM correlate with reduced median survival[10]. In addition to extracellular HA, an intracellular HA has been detected in the nucleus, nucleolus and rough endoplasmic reticulum [11-14]. The source of the intracellular HA is unknown.

HA is synthesized by Hyaluronan Synthase (HAS)[15]. Three isoenzymes of HAS, HAS1 (hCh19), HAS2 (hCh8), and HAS3 (hCh16), have been detected in humans thus far. Each isoenzyme synthesizes different sizes of HA molecules that exhibit different functions[16]. Overexpression of HAS proteins promotes growth and/or metastatic development[17,18]. Little is known about the role of HAS1 in cancer, reflecting a short lifetime of HAS1 transcripts, likely due to the presence of an AU-rich element (ARE) in the 3' UTR gene controlling the half-life of mRNA (ARE database, http:/rc.kfshrc.edu.sa/ared)[19,20].

We have identified differential expression patterns of HAS genes in MM patients. HAS1 is expressed exclusively by circulating MM B cells and HAS2 by BM-localized MM PC, perhaps reflecting different biological imperatives of these two compartments of the MM clone. Both HAS1 and HAS2 are absent from B cells of healthy donors. Furthermore, we identify three novel splice variants of HAS1 designated as HAS1Va, HAS1Vb and HAS1Vc. Expression of HAS1 splice variants is absent from B cells of healthy donors, and in MM and MGUS, is restricted to the B cell compartment. Expression of HAS1Vb, an intronic splice variant, correlates with poor survival in MM patients. This work indicates a potential role for alternative splicing of HAS1 in MM and MGUS.

Chapter 2. 2. Materials and methods

Patient samples

Blood and/or BM samples from 106 patients with MM, and 62 patients with Monoclonal Gammopathy of Undetermined Significance (MGUS) were taken at diagnosis and subsequent clinical visits, after approval from the Health Research Board (U of A) and the Alberta Cancer Board and with informed consent. Blood samples were also obtained from 10 healthy donors.

Tissue and cell preparation

Peripheral blood mononuclear cells (PBMCs) and bone marrow cells (BMCs) were stained and sorted as described by Szczepek et al.[3] The sorted PB CD19$^+$B and BM PC cells with more than 96% purity, and PBMC from 164 (106 MM and 58 MGUS) patients were cllected and resuspended in Trizol (Invitrogen, Carlsbad, CA) or RLT lysis buffer (Qiagen, Mississauga, ON) for total RNA isolation, using either a standard Trizol (GIBCO/BRL) isolation reaction or RNeasy kit (Qiagen) according to the manufacturer's instructions. Total RNA was obtained from cell lines in a similar manner. Cryopreserved cells were never used

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Complimentary DNA for the PCR was reverse-transcribed from total RNA isolated from sorted CD19$^+$ B cells, CD38^{hi}CD45^{lo}PC, non-B, non–PC and T cells or from total mononuclear cells isolated from blood or BM aspirates and cell lines. Total RNA (0.5-1ug) was denatured for 10min. at 70^{0}C followed by annealing with100ng dT15. Next, RNA was reverse-transcribed as described by Keats et al. [21]. The HAS (HAS1, HAS2, and HAS3) gene-specific transcripts were amplified from cDNA obtained through an RT reaction using a reverse and forward primer set designed for each HAS transcript (Table 2-1). For the PCR reaction 1.1ng cDNA was added to 24ul of PCR mix containing 10X PCR buffer, 1ul of 50mM $MgCl_2$, 0.5ul of 10mM dNTPs, 1ul each of 10 mM primers and 5U of platinum Taq (Invitrogen). The PCR cycling parameters were the following: primary denaturation 5min at 94^{0}C, 35cycles of denaturation for 30sec at 94^{0}C, annealing 30sec at 60^{0}C, extension 30sec at 72^{0}C and final extension 7min at 72^{0}C. The samples were either stored at –20^{0}C for later analysis, or immediately processed for capillary electrophoresis on the ABI3100 DNA genetic analyzer (Applied Biosystems (ABI), Foster City, California).

Capillary electrophoresis—DNA fragment analysis

For DNA fragment analysis, 1ul of PCR product was mixed with 12ul of HiDi formamide (ABI) and 1ul of internal size standard GeneScan 500 (ABI). PCR products were then denatured for 4min. at 96^{0}C and after rapid centrifugation; samples were immediately placed on ice for 15 minutes. The electrophoresis conditions were: run voltage 15kVolts, injection voltage 1kVolts, injection duration 10-20sec, run temperature 60^{0}C, laser within power of 12mWatts and run current 100uAmps. The results of were analyzed using GeneScan 3.7 software (ABI).

Cloning and sequencing

RNA for cloning was extracted using an RNAeasy kit (Qiagen). 1ug of RNA was then reverse transcribed as above. HAS1 was amplified in 50ul PCR reaction mix containing 8.8 ng cDNA, 5ul of 1XPCR buffer, 2mM MgSO4, 0.2mM dNTP's, 0.4mM HAS1 cloning primer set (Table 2-1) and 0.5U HiFi Platinum Taq (Invitrogen). The PCR cycling parameters were denaturation for 5min at 94°C, followed by denaturation for 1min at 94°C, annealing for 2min at 60^{0}C and extension at 72^{0}C for 2min for 35 cycles, with a final extension period of 7min at 72^{0}C. HAS1 PCR products were cloned into the TOPO TA cloning system and transformed into TOP 10 competent cells according to the manufacturer's instructions (Invitrogen). To identify colonies containing HAS1 plasmid, individual bacterial colonies were tested by PCR as described above. Positive HAS1/TOPO TA colonies were grown overnight in LB medium. Plasmids were prepared using the Qiagen Plasmid Purification Mini Kit (Qiagen) and sequenced with T7 and M13 primers with the ABI PRISM BigDye V3 Cycle Sequencing Ready Reaction DNA Sequencing Kit (ABI) on the ABI 3100 DNA Genetic Analyzer.

Statistics

After obtaining informed consent, blood samples were collected from MM patients between December 1995 and March 2003. All patient records were reviewed retrospectively to verify the diagnosis of MGUS and MM[22]. Information on diagnosis, patient demographics, baseline staging and clinical features, treatment, and survival were collected. Blood taken at time of diagnosis was available from 58 of the cases; only these cases were used to assess the correlation of HASs with baseline clinical features and survival. The primary analysis assessed the association between expression of the various HAS isoforms and survival. Secondary analyses explored correlations between HAS isoforms and other clinical or laboratory parameters. Categorical variables were compared between two groups using Fisher's exact test. Continuous variables were compared using Student *t* test or the Wilcoxon rank sum test as appropriate. Survival distributions were determined using the Kaplan Meier method and compared using the log rank test. Multivariable analyses and hazard ratios were generated using Cox regression models. Statistical significance was set at a p=0.05 using two-sided analysis.

Particle Exclusion Assay (PEA)

The enzymatic activity of HAS proteins was determined using a PEA as described previously.[23] Sorted CD19$^+$ B cells (2x10^6), obtained from PBMC of 7 MM patients and

3 healthy donors, and CD38^{hi}CD45^{lo}PC (2x10^6) collected from the BM of 3 patients with MM, were cultured in a Poly-L-Lysine (PLL) (1mg/ml; Sigma, Missouri) coated 35 mm culture dish and allowed to recover. After recovery, medium was removed at 4h, 12h, 24h or 48h, and the cells were washed with CMF-PBS. Next, formalin (3%) fixed sheep erythrocytes (50x10^5) in PBS/0.1% BSA were added to the cultured cells. The culture dish was placed on the microscope stage until the fixed red blood cells had settled (30-45 min). Imaging used an Axiovert 100M confocal laser scanning microscope (Zeiss, LSM 510, Germany). As a negative control, the cells were treated with 100ul (500U/ml) of hyaluronidase (HAase, Type-4 from bovine testes; Sigma) for 1h at 37^{0}C prior to adding fixed erythrocytes. Aliquots of the same samples were analyzed by RT-PCR for expression of HAS1 and its variants, as described above.

Intracellular HA detection

PEA was modified and combined with indirect HA staining to verify that the detected pericellular matrix around the cell plasma membrane includes HA molecules. The sorted CD19$^+$B or CD38^{hi}CD45^{lo}PC cells (2x10^5) were cultured and recovered as described above. After the recovery time cells were fixed with 4% paraformaldehyde (PFA) for 15 min at 4^{0}C. To localize HA, the cells were incubated with biotinylated HA binding protein (B-HABP) Seikagaku America, USA (BioLynx, San Antonio, TX)) (2ug/ml in PBS/1% BSA over night at room temperature). Alternatively, B-HABP was detected with streptavidin Alexa 594 (Molecular Probes, Eugene OR) 1:500 dilution for 2 h at RT. As a negative control sorted cells were treated with 500U/ml hyaluronidase for 1h at 37^{0}C before and after fixation or B-HABP were pre-incubated with 100 μg Hyaluronan

(Seikagaku America, USA). The specificity of streptavidin was determined by staining the cells with streptavidin Alexa 594 only. The cells were examined with an Axiovert 100M LSM 510.

To detect intracellular HA, CD19$^+$B cells were sorted and mobilized on a PLL coated dish as described above. Cells were treated with HAase for 2h at 37^{0}C, washed with PBS, fixed/permeabilized with 4% PFA/0.03% Saponin and incubated with 2ug/ml B-HABP over night at 37^{0}C. Intracellular HA was detected by streptavidin Alexa 594 as described above.

Cell lines and antibodies

The MM cell line RPMI 8226 was generously supplied by S. Treon (The Dana-Farber Cancer Institute). KMS-12-BM and KMS-12-PE were generous gifts from T. Otsuki (The Kawasaki Medical School). The rabbit polyclonal antibody recognizing HAS1 and variants was produced by Washington Biotechnology (Baltimore, MD) to the following peptide sequence: ^{135}GNRAEDLYMVDMFRF^{150}. The polyclonal serum was affinity purified and the specificity was tested by ELISA and Western blotting, the latter of which was performed on protein lysates obtained from the CCL 110 cell line expressing all isoenzymes of HAS (results not shown).

Western blotting

The cells, RPMI 8226, KMS-12-BM and KMS-12-PE, were lysed at 5 × 10^6–10^7 cells/ml in RIPA buffer with protease inhibitors. Equal amounts, 2ug of total proteins obtained from cell lysates, were separated by a 5%stacking/12% separating SDS-PAGE and transferred onto Immobilon^{TM}-NC membranes (Millipore, Bedford, MA). Nonspecific binding was blocked by 5% BSA/0.1% Tween 20 PBS, blocking buffer, overnight at 4^0C. The membranes were incubated with the anti-HAS1 serum (1:500), pre-immune serum (1:500), and anti-HAS1 serum incubated with five-fold excess blocking peptides, overnight at 4^0C. Next membranes were incubated for two hours with an anti-rabbit HRP-IgG (1:20000) (Jackson ImmunoResearch Laboratories, Inc.). The immune complexes were visualized using ECL reagent according to the manufacturer's instructions (BioBar, Amersham Biosciences, Canada).

Chapter 2. 3. Results

Differential expression of HASs in MM or MGUS PBMC B cells and BM PC

The MM clone includes both BM plasma cells and circulating CD19$^+$ B cells previously shown to be clonotypic[3,4,7,24]. The expression of HASs was examined in CD19$^+$B cells obtained from patients with MM, MGUS, and healthy donors, as well as in CD38^{hi}CD45^{lo}PC sorted from MM BM. Using RT-PCR and DNA fragment analysis, we detected ubiquitous expression of HAS3 in all analyzed patients and healthy donors (Figure 2-1C; Table 2-2). In contrast, cell-type specific expression patterns of HAS1 and HAS2 were detected in malignant B and PC respectively. CD19+B cells from 7/13 MM patients expressed HAS1, while HAS2 was detected in CD38^{hi}CD45^{lo}PCs from 11/11 MM patients (Table 2-2; Figure 2-1B,C). No HAS2 was detectable in sorted PC from two and unsorted BMC from three lymphoma patients with uninvolved BM.

Expression of HAS2 was absent in MM CD19^{+}B cells even after the precipitation of 25ul of PCR products, a method to increase the sensitivity of detection for templates with low abundance. Similar to MM B cells, CD19^{+}B cells obtained from the PBMC of 4 patients with MGUS expressed HAS1 and HAS3 but not HAS2 (Figure 2-1A, B; Table 2-2). Precipitation of PCR products revealed weakly detectable expression of HAS1 in CD38^{hi}CD45^{lo} PCs from only 3/11 MM patients (Table 2-2). No HAS1 or HAS2 expression was detectable in non-PC obtained from the same set of samples from which CD38^{hi}CD45^{lo}PCs were isolated. Furthermore, no HAS1 was detectable in non-B cells (CD19^{-} PBMC) in the same samples from which MM and MGUS CD19^{+}B cells were isolated, or from sorted T cell populations obtained from PBMC of 4 MM patients. Overall, strong HAS1 expression in MM and MGUS was restricted to CD19^{+}B cells, while HAS2 is restricted to MM PC.

To determine whether or not expression of HAS1 is unique to MM and MGUS CD19^{+}B cells, we analyzed CD19^{+}B cells from the PBMC of 10 healthy donors. B cells from healthy donors expressed only HAS3 transcripts (Table 2-2; Figure 2-1C). HAS1 and HAS2 were undetectable in these cells, even after precipitation/analysis of 25ul of PCR products.

Furthermore, HAS1 expression was assessed for 82 MM and 58 MGUS patients. Archived PBMC were used, justified by our demonstration that in MM and MGUS, expression of HAS1 is restricted to the CD19$^+$B cells in PBMC and was undetectable in non-CD19$^+$ PBMC (Table 2-2). Table 3 shows HAS1 expression for PBMC from 58% of MM and 74% of MGUS patients.

Novel aberrant splice variants of HAS1 in sorted MM and MGUS B cells

Using RT-PCR and DNA fragment analysis, we identified three splice variants of HAS1, designated as HAS1Va, HAS1Vb and HAS1Vc, in MM and MGUS CD19$^+$B cells (Figure 2-1A). We analyzed the expression of these variants in sorted CD19$^+$B cells isolated from PBMC of 13 MM patients and CD38^{hi}CD45^{lo}PC obtained from BM of 11 MM patients. HAS1Va was expressed in CD19$^+$B cells from 8/13 MM and 4/4 of MGUS patients (Table 2-2). From the same set of samples HAS1Vb was expressed in 7/13 MM patients, while no expression of these transcripts were detected in CD19$^+$B cells obtained from 4 patients with MGUS (Table 2-2). HAS1Vc was expressed in 6/13 MM and 1/4 MGUS patients (Table 2-2). MM PC from only 1/11 patients expressed HAS1Va and HAS1Vb, and none expressed detectable HAS1Vc (Table 2-2). Additionally, no splice variants of HAS1 were detectable in CD19 negative fractions of PBMC (non-B cells) from 9 MM patients or in non-PC populations (BMC remaining after gating for CD38^{hi}CD45^{lo}PC) obtained from three MM BM samples. No HAS1 variants were detectable in CD19$^+$B cells from 10 healthy donors or in sorted T cells of 4 MM patients. Thus, the expression of one or more HAS1 variants was restricted to MM and MGUS CD19$^+$B cells. This allowed us to examine recurrent expression of HAS1 splice variants in unfractionated PBMC obtained from 82 MM and 58 MGUS patients. HAS1Va transcripts were detected in 68% of MM and 74% of MGUS patients, while HAS1Vb was identified in 46% of MM and 41% of MGUS PBMC (Table 2-3). HAS1Vc was detected in 33% of MM and 9% of MGUS PBMC (Table 2-3). HAS3 was ubiquitously expressed in all samples tested providing a control for RNA integrity. This analysis demonstrates that the majority of MM and MGUS patients express one or more HAS1 variants in various combinations.

Longitudinal analysis of HAS1 and variants in MM patients

Longitudinal monitoring of HAS1 and its variants was performed for PBMC from 18 unselected MM patients. At the time of diagnosis 65% (11/18) of MM patients expressed HAS1 alone or in combination with one or more novel variants, while 71 % (10/14) of MM patients expressed these genes at the time of relapse. No distinct expression pattern was observed in patient samples obtained during disease progression or remission, possibly reflecting treatment of many patients with prednisone and/or dexamethasone, which are known to play a significant role in tissue specific regulation, activation and/or suppression, of HAS genes and production of HA[25,26].

Cloning and sequencing of HAS1 variants

Using RT-PCR, we amplified HAS1Va and HAS1Vb cDNA fragments from CD19^{+}B cells of three MM patients and HAS1Vc cDNA fragments from one MM patient. The variants of HAS1 were amplified using hot start PCR and an exon-intron spanning primer set (Table 2-1). The PCR products were cloned into the TOPO TA vector and positive sub-clones were identified by PCR with HAS1 gene specific primers. Plasmids isolated from positive clones were sequenced. Sequences were identified through alignment with the published sequence of human HAS mRNA (gi:4504338; NCBI). This analysis identified HAS1Va as a result of complete deletion of exon 4 leading to a frameshift after the deletion and creation of a premature termination codon (PTC), 56 nucleotides downstream of the deletion (Figure 2-2A). HAS1Vb is the result of partial retention of intron 4 (59 bp) at the 5' end of exon 5 and deletion of the entire exon 4. These aberrations lead to a frameshift after deletion of exon 4 that creates a PTC 93 nucleotides downstream of retained intron 4, at the beginning of exon 5 (Figure 2-2B). HAS1Vc results from the retention of 26 nucleotides of intron 4 at the 3' end of exon 4, causing truncation of the HAS1 transcripts and insertion of PTC at the 3' end of exon 4 (Figure.2-2C). For all three variants, the start codon and the conserved sequence of the glycosyltransferase motif was present in the aligned cDNA sequences, consistent with the evidence below that HAS1 variants retain the ability to synthesize HA.

Cloning, sequencing, and alignment analysis of HAS1 variants from CD19^{+}B cells of one MM patient revealed a point mutation (a possible single nucleotide polymorphism—SNP) on exon 3 of the HAS1Va transcripts (Figure 2-2A). HAS1Va from this MM patient had the nucleotide T instead of C in this position (7760C>T). The detected point mutation was confirmed through twice sequencing both strands (plus and minus strands) of HAS1Va cDNA, in a triplicate sequencing reaction by triplicate runs. Also transcripts were amplified using HiFi Platinum Taq which has proofreading ability. The point mutation 7760C>T was absent in the HAS1^{FL}, HAS1Vb and HAS1Vc transcripts obtained from the same B cell population of the same patient, suggesting that the HAS1Va and the HAS1/ HAS1Vb/HAS1Vc group were derived from different alleles.

Correlated expression of intronic variants HAS1Vb and HAS1Vc

This analysis incorporated 74 randomly selected MM cases. Pairwise relationships between various HAS's were assessed with Fisher's exact test. This analysis showed that expression of HAS1Vb and HAS1Vc intronic splice variant transcripts in MM patients was significantly correlated (p=0.01). Sixteen MM patients co-expressed both variants, 33 expressed neither variant, 16 expressed only HAS1Vb, and 9 expressed only HAS1Vc. However, the correlation between HAS1Vb and HAS1Vc is only moderate; the population of HAS1Vb expressors only partly overlaps the population of HAS1Vc expressors. This result was similar when only 58 diagnosis samples were considered (p=0.03). There was no statistically significant relationship in expression among the other HAS1 variants.

HAS1Vb expression by MM B cells correlates with reduced survival

Expression of HASs was analyzed in PBMC obtained from 58 MM patients at time of diagnosis. The univariate analysis of these cases showed that expression of HAS1Vb in MM patients was most strongly correlated with a shorter survival (HR=2.6, 95%CI, 1.4-4.8 p=0.001, Fig.3). Associations between HAS1Va (p=0.048) and HAS1^{FL} (p=0.12) and poor survival were of borderline or non-statistical significance. There was no association between expression of HAS1Vc and poor survival (p=0.66).

Expression of HAS1 variant protein and HA synthesis in MM cells

Protein expression was evaluated by Western blotting in MM cell lines (RPMI 8226, KMS-12-BM, KMS-12-PE) (Figure 2-4). Bands corresponding to the HAS1Va (35.9KD) and HAS1Vb (39.5KD) were detected in all three cell lines although expression of HAS1Vb was weak in RPMI 8226. Overall, expression levels of HAS1 proteins were low. In parallel, expression of HAS1, HAS1Va and HAS1Vb was consistent with the protein expression in aliquots of the same cells (Figure 2-4D); HAS1Vc was undetectable.

Enzymatic activity of the HASs was detected using a Particle Exclusion Assay (PEA) (Figure 2-5A,B). HA matrix was detected around MM CD19$^+$B cells after 48 hours of culturing. No matrix accumulation was observed around MM BM PC or B cells obtained from healthy donors (not shown). Hyaluronidase treatment removed the deposited matrix around the cell plasma membrane of MM CD19$^+$B cells (Figure 2-5C), confirming the presence of HA (Figure 2-5A,B). The existence of HA molecules in the pericellular matrix detected by PEA was verified by incubating cells with B-HABP (Figure 2-6). Using B-HABP, we also detected intracellular HA in permeabilized MM CD19$^+$B cells (Table 2-4). Intracellular HA in these cells is abundantly distributed around the perinuclear compartment of the cells as well as along the cell cytoskeleton (Figure 2-6F).

To clarify the role of HAS1 and its variants in the enhanced extracellular and intracellular production of HA molecules by the $CD19^+$ B cells of patients with MM, we have correlated the expression of HASs with the production of extra- and/or intracellular HA in these cells using PEA, HA staining and RT-PCR. Although the patterns are complex, analysis of B cells from 15 MM patients (Table 2-4) shows that synthesis of HA is detectable only by those populations of B cells expressing HAS1 variants. The potential contribution of hyaluronidases has not yet been assessed. Overall, HAS1Va appeared to be associated with synthesis of extracellular HA and HAS1Vb with synthesis of intracellular HA. Expression of HAS1 with HAS3 appeared insufficient for synthesis of either form of HA.

Chapter 2. 4. Discussion

This study shows that circulating MM and MGUS B cells express HAS1 and a family of alternative splice variants, and that alternative splicing of the HAS1 gene in MM B cells predicts for reduced survival. The HAS1 gene and its variants are largely absent from non-B cells, as well as from BM-localized MM PC, perhaps reflecting differentiation events within the MM clone. HAS1Vb is the result of abnormal intronic splicing events, which appear to involve the activation cryptic splice sites within HAS1. Similar splicing patterns have been observed for other genes which are associated with malignant phenotypes[27-30]. HAS1 and its variants are absent from B cells of healthy donors, and from non-B or T cells from blood and BM of MM patients. Analysis of normal human tissues showed upregulation of HAS1 only in lung tissue (NCBI GEO profiles), consistent with the view that it may be predominantly expressed in malignant cells[31]. No HAS1 variants have been reported in the GEO database. Expression of HAS1 variants appears to be exclusive to malignant B lineage cells in MM, MGUS and Waldenstrom's macroglobulinemia[32].

In MM, expression of HAS1 variants is restricted to MM CD19$^+$B cells. BM CD38^{hi}CD45^{lo}PC expressed HAS1Va and HAS1Vb, at very low levels, in only 1/11 MM patients. HAS1Vc transcripts were undetectable in MM PC. HAS1 variants were absent from T cells obtained from MM PBMC of 4/4 patients, and non-B cell (CD19-negative populations) from the PBMC of 9/9 MM patients. Thus, HAS1 variants are consistently detected in sorted MM CD19$^+$B cells but not in BM CD38^{hi}CD45^{lo}PC or in other cell populations comprising PBMC of MM and MGUS patients, or BMC of MM patients. This suggests biologically important changes in MM gene expression profiles as malignant B cells differentiate to PCs. Calabro et al. through their study suggested expression of HAS1 transcripts in MM BM mesenchymal progenitor cells[33]. However, freshly isolated MM BM mesenchymal progenitor cells do not express HAS1 transcripts as shown by Grskovic et al. who demonstrated that freshly isolated or cultured BM progenitor or non-progenitor cells lack HAS1 transcripts[34]. Discrepancies between these studies may reflect differences between isolation and culturing procedures.

Recently, it was reported that expression of HAS1 and HAS2 in HR-3Y1 cells corresponds to the degree of malignant cell transformation[35]. Up-regulation of the HAS1 gene was observed in highly malignant cells transformed with v-src and/or with v-fos [35].

Although alternative splicing is a normal event contributing to protein diversity in humans, more than a dozen human cancers are associated with abnormalities in alternative splicing, including intronic splicing. One cause of aberrant splicing is mutation, the consequences of which are exon skipping and/or intron retention [36-38]. Our study suggests that HAS1 and its variants, particularly HAS1Vb, may contribute to early myelomagenesis since these transcripts are detected individually or in combination with other variants in PBMC obtained from the majority of MM and MGUS patients at the time of diagnosis.

Longitudinal expression analysis of HAS1 and variants in PBMC of 18 unselected MM patients showed sporadic expression of HAS1 and its variants throughout disease, with expression in a majority of MM patients at diagnosis (65% of patients) and in relapse (71.4% of patients). This may reflect treatment of many patients with corticosteroids, known to inhibit some HASs and production of HA[25,26,35]. Thus, expression of the HAS1 family genes, mainly HAS1Vb, appears to characterize circulating MM cells in the blood of patients at both, early and late stages of the disease. Furthermore, the observation that HAS1 and variants are found in PBMC of MM patients but not healthy donors, together with the association between HAS1Vb and poor survival, suggests that these alternatively spliced HASs are upregulated at early stages of malignant transformation and may contribute to the spread of malignancy.

Alignment analysis of HAS1 variants with HAS1^{FL} demonstrated that the complete motif of glycosyltransferase is retained, consistent with demonstration that expression of HAS1 and/or HAS1 variants is required for the production of HA. Expression of HAS1Va, which was detected in circulating B cells of 8/13 MM patients correlated with poor survival (p=0.048), and may be required for synthesis of extracellular HA. A point mutation 7760C>T detected on the highly conserved exon 3 of HAS1Va could promote activation of cryptic splice sites and consequently mediate the splicing and truncation of HAS1 since this mutation is located in the vicinity of splicing signals of the gene. The occurrence of the point mutation 7760C>T in HAS1Va transcripts and its absence in HAS1^{FL}, HAS1Vb and HAS1Vc obtained from the same patient suggest the presence of a new variant allele of HAS1 in MM patients. Currently, we are cloning and sequencing HAS1 gene from genomic DNA of MM patients to clarify whether or not the changes detected on HAS1Va transcript represent mutation or polymorphism.

HAS1Vb, which correlates strongly with poor survival (p=0.001), partially retains intron 4 most likely through the activation of cryptic 5' and/or 3' splicing sites. Previous analyses of other genes have shown that this type of aberration, the retention of introns during splicing, is uncommon (6%) and it is often associated with short introns[36]. In most cases, partial retention of introns appears to be characteristic of genes associated with a malignant phenotype[27-29,39]. As shown here, intronic splicing of HAS1 also correlates with the malignant phenotype. The alternatively spliced HAS1Vb transcripts are expressed in circulating B cells from a majority of MM patients, at diagnosis and relapse. Alternative splicing of HAS1Vb causes a frameshift and insertion of PTC downstream of the deleted exon and after the retained part of intron, leading to a truncated protein. Moreover, HAS1Vb may be required for synthesis of intracellular HA; only MM B cell populations expressing HAS1Vb produced intracellular HA. In addition, protein encoded by HAS1Vb, similar to the MDM2 splice variant, may form dimers with wild type HAS1 and thus alter normal functioning of the full length HAS1 protein[39,40]. HAS1Vc, another intronic splice variant of HAS1 that retains exon 4, shares similar but not identical splicing and expression patterns with HA1Vb. No correlation was found between HAS1Vc and patient survival. This suggest that HAS1Vc may act in a dominant-negative manner to compromise normal functioning of HAS1.

All three HAS1 splice variants are truncated. However, alignment and protein motif screening analysis (Prosite-MotifScan) showed that all three variants of HAS1 retain the complete motif of glycosyltransferase which carries out the synthesis of HA molecules. Protein expression of HAS1 variants is supported by the Western blot analysis conducted on MM cell lines. Furthermore, HAS1Va and/or HAS1Vb, perhaps in concert with HAS3, appear to be required for, respectively, synthesis of extracellular HA matrix or intracellular HA by MM B cells; B cells obtained from healthy donors expressing only HAS3 and MM PC, which express HAS2 together with HAS3 but lack HAS1, are unable to synthesize extracellular/intracellular HA. Further, expression of HAS1, HAS1Va and HAS1Vb, and detection of an HA matrix correlates with cell motility. Among the B lineage cells in MM, only MM B cells include a motile subset. Upregulation of HAS1 and variants may contribute to the spread of MM independently or in concert with dowregulation of hyaluronidases.

Recently, the interaction between endogenous HA synthesis and multidrug resistance has been documented[41]. Ex- and in- vivo MM B cells, the only components of the MM clone to express the HAS1 family, are highly drug resistant[7]. Baumgartner et al. showed that hyaluronidase treatment improved the effects of various chemotherapeutic agents[42]. Thus, synthesis of extracellular HA by MM B cells may impact disease biology by contributing to drug resistance.

Based on alignment and Western blot analysis, transcripts of HAS1 and its variants appear to be translated to form functional proteins. However, for the HAS1 variants, deletion of the entire exon 4 and most of the exon 5 could alter their proper membrane folding, compromising translocation of HA into the ECM and potentially distributing HA molecules into an interior cellular compartment, as detected here (Figure 2-6F). A similar localization of intracellular HA in Ras-transformed cells has been reported by others[12]. We believe that intracellular HA detected in MM B cells is produced by one or more HAS1 variants, particularly HAS1Vb which is associated with poor survival. Strong perinuclear localization of intracellular HA branches out from the perinuclear compartment toward the cell plasma membrane. Identification of this type of intracellular HA staining pattern suggests that these molecules may also contribute to the process through which malignant cells maintain cellular architecture. Finally, it is intriguing to speculate that the intracellular HA molecules produced by HAS1Vb may through HA binding modulate the function of RHAMM, which is overexpressed and contributes to mitotic abnormalities in MM[43-45].

Chapter 2. 5. Acknowledgments

We thank the many patients at the Cross Cancer Institute and University of Alberta for their generous donations and their participation in this study. The skilled assistance of Viet Hoang, Juanita Wizniak and Tara Tiffinger is gratefully acknowledged.

Table 2-1. Nucleotide sequences of primer sets used in the study

GENES	PRIMER SETS	
	Reverse	**Forward**
HAS1	5’ VIC-GGGCTTGTCAGAGCTACTT	5’ AGGGCGTCTCTGAGTAGCAG
HAS2	5’ VIC-CCTCATCTGTGGAGATGGGT	5’ TCCCAGAGGTCCACTAATGC
HAS3	5’ VIC-CATCCAGGTGTGCGACTCTG	5’ CGCTGCTCAGGAAGGAAATC
CD19	5’ FAM-TACTATGGCACTGGCTGCTG	5’ CACGTTCCCGTACTGGTTCT
RHAMM	5’ FAM-TGACAAAGATACTACCTTGCCTGCT	5’ CAGCATTTAGCCTTGCTTCCATC
HAS1 CLONING PRIMERS	5’ GCCTTCGCCCTGCTCATCCTG	5’ GTAGAACAGACGCAGCACA

For these primer sets, the reverse primers were labeled at their 5’ ends with 6-carboxyflourescein (FAM) or VIC (chemical name not disclosed; ABI). Primers were designed using the "Primer 3" or "Gene Tool" programs based on the published cDNA sequences of the HASs, CD19 and RHAMM.

Table 2-2. HAS1 gene expression is restricted to MM and MGUS B cells

Sample type	Patient	HAS1Va	HAS1Vb	HAS1Vc	HAS 1	HAS 2	HAS 3
MM $\text{CD38}^{hi}\text{CD45}^{lo}$ PC **(n=11)**	#1-8	-	-	-	-	**11/11**	**11/11**
	#9	-	-	-	+		
	#10	+	+	-	+		
	#11	-	-	-	+		
MM CD19^{+} B **(n=13)**	#12	-	-	-	+	**0**	**13/13**
	#13, 18	-	+	-	+		
	#14, 16	+	-	+	-		
	#15	-	+	-	-		
	#17	-	+	+	+		
	#19, 20	+	-	-	+		
	#21	+	+	-	-		
	#22, 23	+	+	+	-		
	#24	+	-	+	+		
MGUS CD19^{+}B **(n=4)**	#25-27	+	-	-	+	**0**	**4/4**
	#28	+	-	+	-		
Healthy donors, CD19^{+} B cells **(n=10)**		**0**	**0**	**0**	**0**	**0**	**10/10**

Expression profiles of HAS genes and HAS1 novel variants in CD19^{+}B cells from PBMC of MM/MGUS/healthy donors, and from $\text{CD38}^{hi}\text{CD45}^{lo}$ PCs from BM aspirates of MM patients. Expression profiles of HASs in MM PBMC non-B cells (CD19 negative fraction of PBMC cell populations obtained from the same samples from which CD19^{+}B cells were isolated), MM BM non-PCs (fraction of non-PC populations obtained from the same set of samples from which $\text{CD38}^{hi}\text{CD45}^{lo}$PCs were isolated) and MM T cells are not shown in the table since these samples express HAS3 only. No HAS1, HAS1 variants or HAS2 was detected in these samples. The table indicates the number of patients analyzed for HAS gene expression; "+" and "-" indicate positive and negative expression of the indicated HAS transcripts. HAS2 expression was analyzed in sorted PC obtained from 2 and unsorted BMC from 3 lymphoma patients with uninvolved BM; no HAS2 was detected in any of these samples using RT-PCR and DNA fragment analysis/GeneScan software. Expression of CD19 in B cells obtained from patients and/or healthy donors and RHAMM expression in the BM PC were used as positive control reactions to validate the integrity of RNA. All analyzed samples expressed CD19 (MM and healthy donor B cells) and RHAMM (MM PC) transcripts (not shown). As a negative control for each PCR sample, reactions were always run in the absence of reverse transcriptase at the RT step (not shown).

Table 2-3. HAS1 and aberrant novel variants are expressed in PBMC obtained from the majority of MM and MGUS patients

Sample Type	**HAS1Va**	**HAS1Vb**	**HAS1Vc**	**HAS1**
MM PBMC (n=82)	68%	46%	33%	58%
MGUS PBMC (n=58)	74%	41%	9%	74%

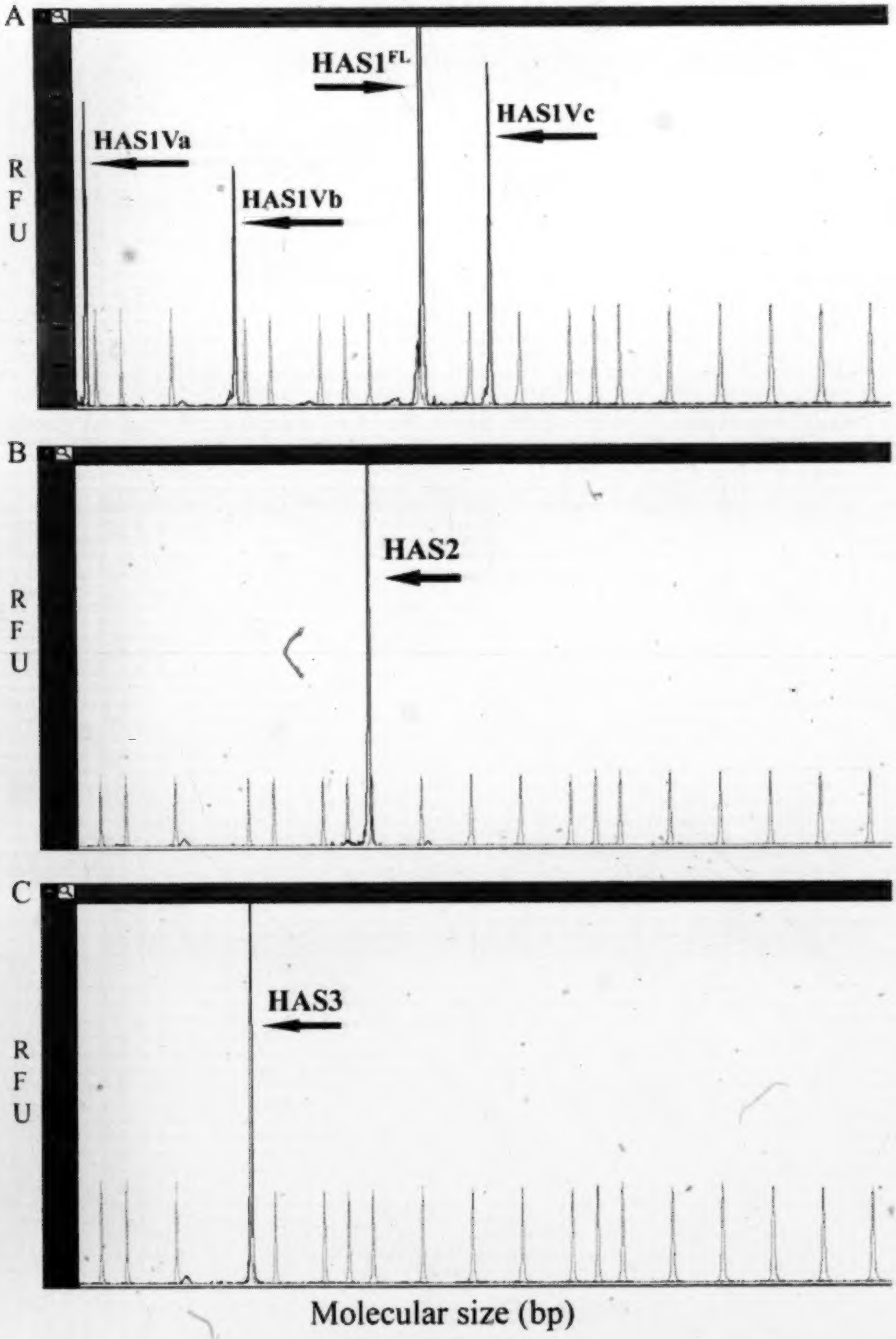
Numbers in the table indicate percentage of patients whose PBMC expressed HAS1 and its novel variant. Results were obtained by RT-PCR/DNA fragment analysis and data analysis was conducted using GeneScan software. HAS1Vc expression is not significantly different between MM and MGUS.

Table 2-4. Expression of HAS1 variants correlates with production of extracellular and/or intracellular HA

Number of patients	HAS RNA expression pattern	HA production
2	HAS1Va, HAS3	Extracellular HA matrix
3	HAS1Va, HAS1, HAS3	Extracellular HA matrix
3	HAS1Va, HAS1Vb, HAS1, HAS3	Extracellular HA matrix & intracellular HA
2	HAS1Vb, HAS1, HAS3	Intracellular HA & very weak extracellular HA
3	HAS1, HAS3	No HA production
2	HAS3	No HA production

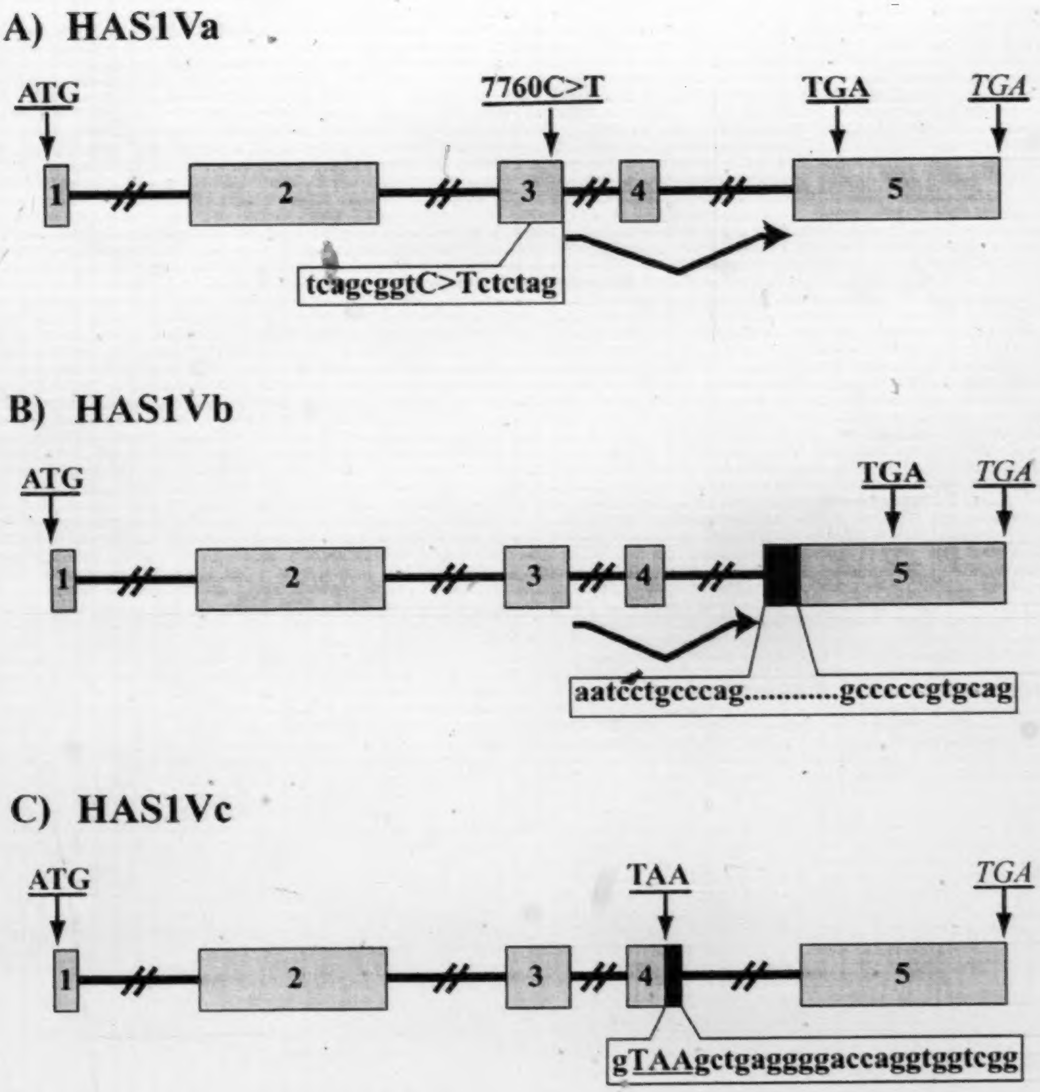
This table summarizes the expression of HAS1, its novel variants and HAS3 genes in PBMC obtained from 15 MM patients. In conjunction with gene expression analysis, we conducted PEA and HA staining on PBMC B cells obtained from aliquots of the same populations of cells from MM patients to evaluate the potential role of HAS1 variants in the enhanced production of extracellular and intracellular HA. "No Hyaluronan production" means that cells did not exhibit extracellular HA matrix around their plasma membrane or intracellular HA as detected by HA staining. However, HA staining demonstrated that the cells without extracellular HA matrix did show cell surface HA staining. Whether these HA molecules are located in or inner lip of plasma membrane has not been conclusively shown.

Figure 2-1. HAS genes are expressed in MM



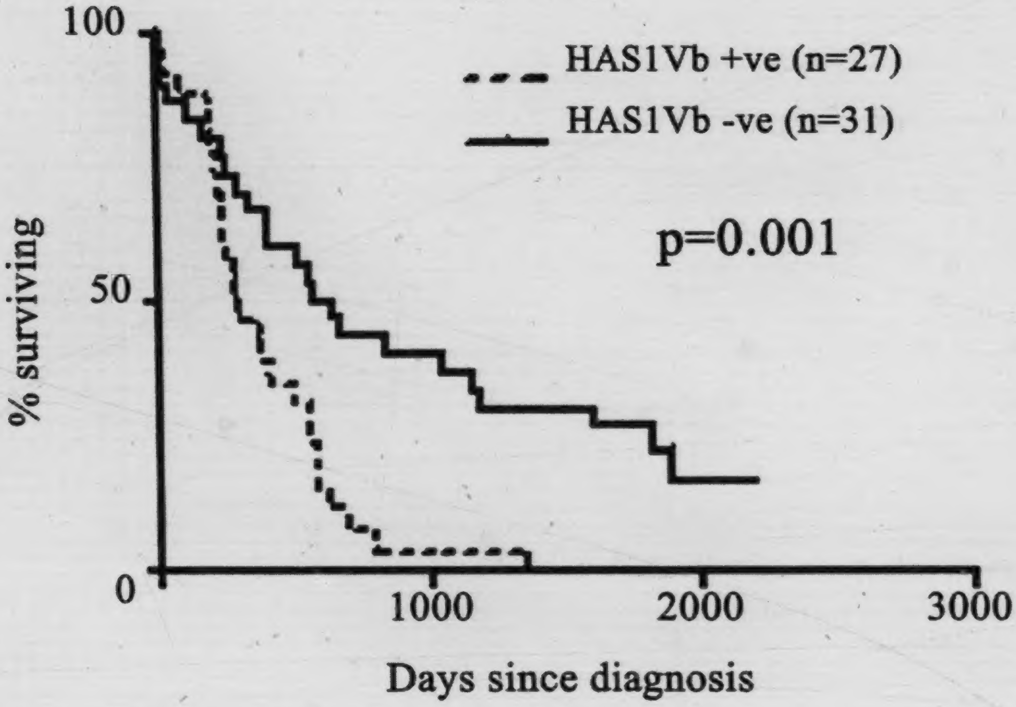
Representative GeneScan electropherograms for DNA fragment analysis of HAS gene expression are shown. PCR products were obtained by RT-PCR amplification of fragments of HAS genes and aberrant variants. The x-axes represent molecular size (bp) of PCR product and the y-axes indicate Relative Fluorescent units (RFU). The arrows indicate product peaks; faded peaks represent internal size standard peaks of LIZ 500 (ABI).

Figure 2-2. MM B cells express aberrant splice variants of HAS1



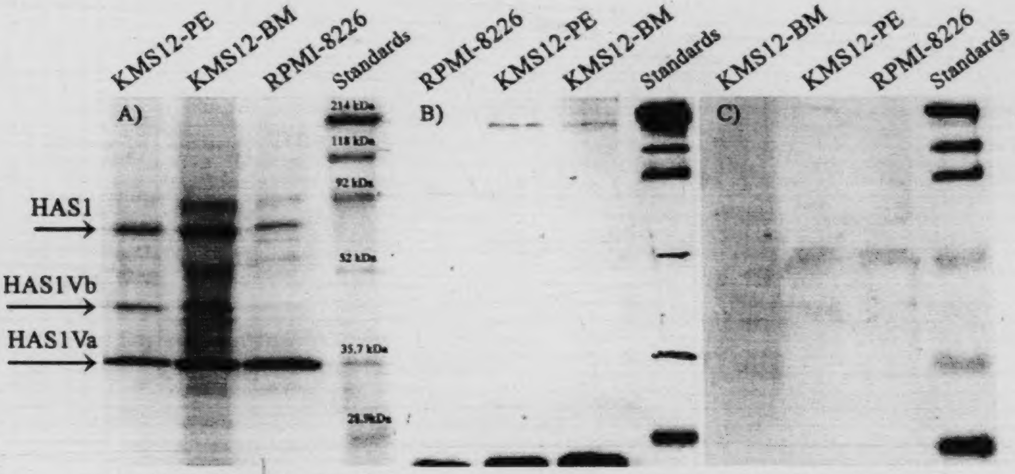
A schematic representation of HAS1Va (A), HAS1Vb (B), HAS1Vc (C). Gray boxes represent exons, while black boxes are retained fragments of intron 4. Introns are shown with solid lines. Original stop codons of each novel variant are marked with italic letters, while bold uppercase letters indicate start codons and PTC. (A) HAS1Va—point mutation 7760C>T detected in this novel variant transcripts is shown in bold, uppercase letters; PTC is located 56 nucleotides downstream of deleted exon 4. (B) HAS1Vb is the result of deletion of the entire exon 4 and partial retention of intron 4 (59 bp, 12 first and last nucleotides of retained introns are shown) at the 5' end of exon5. These aberrations harbored PTC 93 nucleotides downstream of the retained intron 4. (C) HAS1Vc, intronic splice variant is similar to HAS1Vb and is the result of the retention of 26 nucleotides of intron 4, causing insertion of a PTC, TAA, at the 3' end of exon 4. The twenty six nucleotides of retained intron 4 are shown on the figure PTC is shown in bold uppercase letters.

Figure 2-3. HAS1Vb expression by MM B cells correlates with poor survival



Kaplan-Meier survival distributions of MM patients with (dashed curve) or without (solid curve) detectable HAS1Vb in the PB at time of diagnosis. HR=2.6, 95% Cl, 1.4-4.8, p=0.001 using the log rank rest.

Figure 2-4. Expression of HAS1 and novel variant genes and proteins in MM cell lines



anti-HAS1 polyclonal antibody

blocking peptide

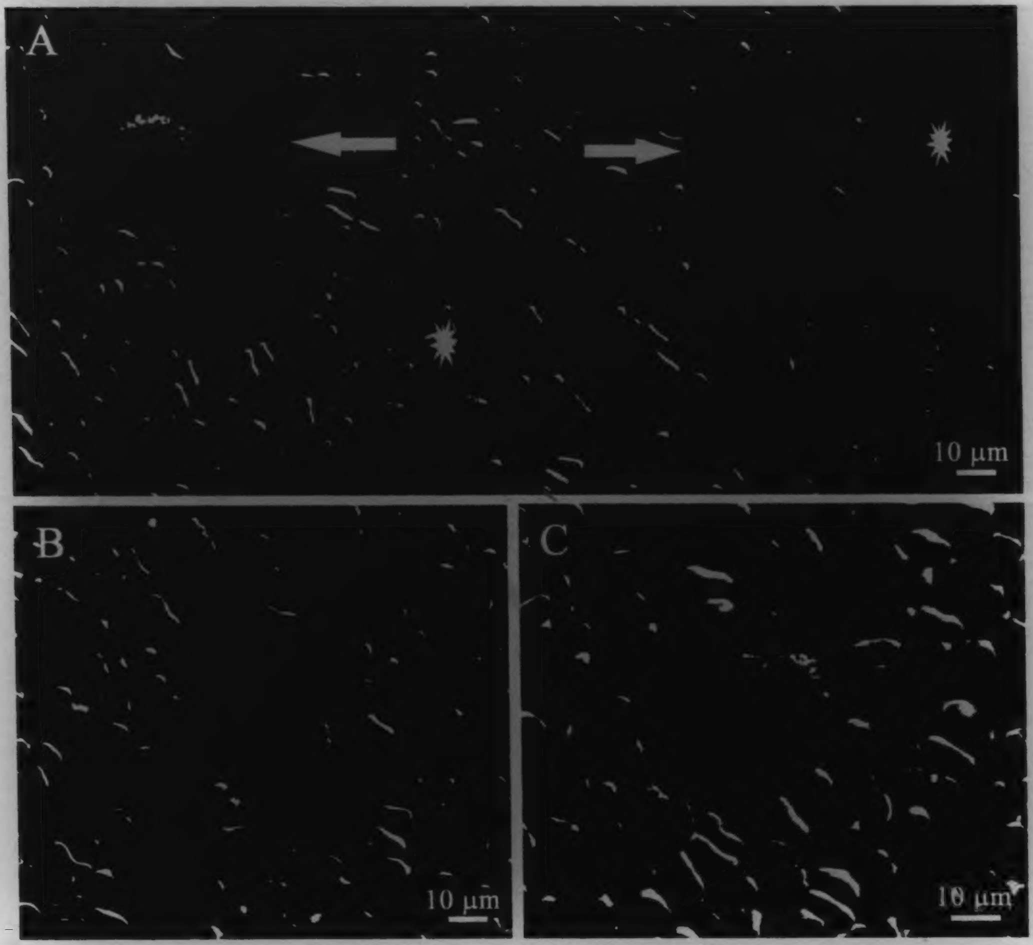
pre-immune serum

D)

Cell lines	HAS1Va	HAS1Vb	HAS1
KMS12-PE	+	+	+
KMS12-BM	+	+	+
RPMI-8226	+	−	+

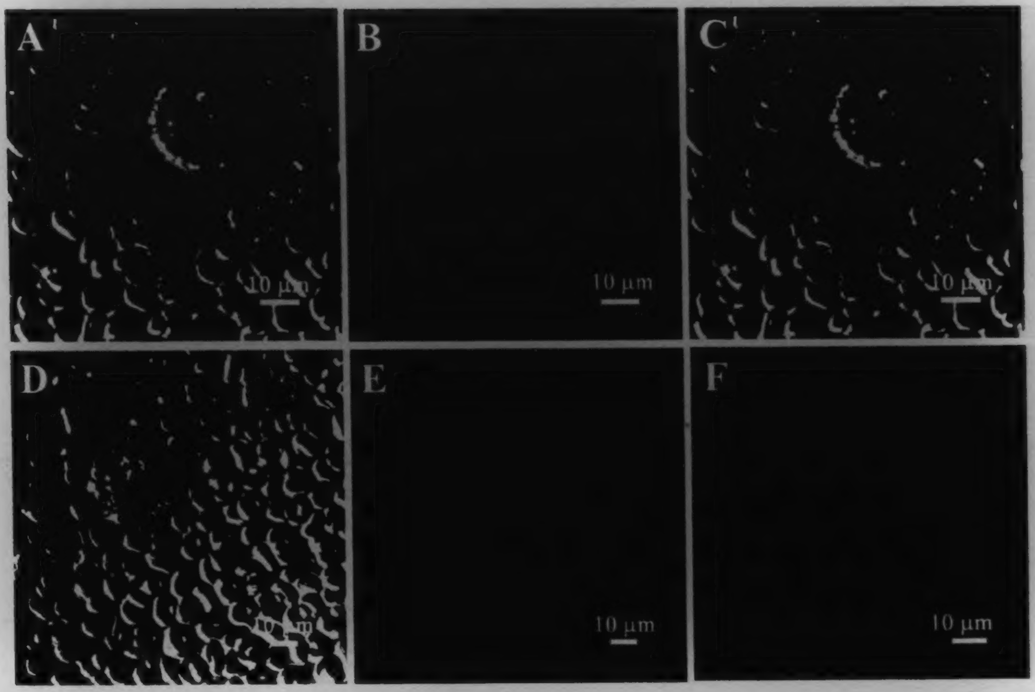
Figure 2-4 represents two separate experiments: A, B, C) Western blot analysis. Protein lysates of MM cell lines were separated on SDS-PAGE, blotted onto nitrocellulose, and probed with A) anti-HAS1 antibodies B) pre-incubated anti-HAS1 with blocking peptide and C) pre-bleed. Arrows identify HAS1Va— ~35.9KD, HAS1Vb— ~39.5KD and HAS1— ~65KD bands. The size of HAS1Va (~35.9KD) and HAS1Vb (~39.5KD) proteins were predicted using the ExPASy Molecular Biology Server. The anti-HAS1 Ab specificity was evaluated by pre-incubating anti-HAS1 serum with the blocking peptide overnight before probing the membrane. The extra bands presented on the blot most likely are bands corresponding to HAS1 proteins encoded by other yet to be identified variants and/or these bands represent the HAS proteins subjected to postranslation modifications and glycosylation. D) RT-PCR DNA fragment analysis. Transcript levels of HAS1 and novel variants were measured in three MM cell lines, RPMI-8226, KMS-12-PE and KMS-12-BM. Total RNA for RT-PCR and cell lysate for Western blot analysis were obtained from same cell-culture for each cell line.

Figure 2-5. HAS1 expressing MM B cells synthesize a pericellular HA matrix



The HA pericellular matrix around the cell was visualized by the addition of fixed erythrocytes to sorted $CD19^{+}$B cells from MM (n=15) and healthy donors (n=3) and from MM $CD38^{hi}CD45^{lo}$PC (n=3) in short-term culture. Fixed erythrocytes were physically excluded from the areas surrounding a cell that had synthesized an HA pericellular matrix. HA matrix was detected only around MM $CD19^{+}$B cells. MM BM $CD38^{hi}CD45^{lo}$ PC and $CD19^{+}$ B cells obtained from healthy donors did not exhibit an HA matrix around their plasma membranes (results not shown). Furthermore, no HA matrix was detected around the MM $CD19^{+}$B cells at 4h and 12h of culturing, however 24h later, a small amount of HA matrix was detected around some MM $CD19^{+}$B cells, while other B cells in the culture did not exhibit an HA matrix. The size of an HA matrix significantly increased around some MM $CD19^{+}$ B cells 48h after culture (A- arrows; B), while other $CD19^{+}$B cells did not develop an HA matrix (A- stars). In the culture some cells were characterized by a prominent coat of HA at one edge of the cell while the opposite edge of the same cell exhibited lesser amounts of HA matrix (B). This type of distribution of HA matrix around the cells results from cell motility. Motile cells exhibit a prominent HA halo at their trailing edge and a lesser pronounced halo at their leading edge. After hyaluronidase treatment no HA matrix was detected around MM $CD19^{+}$ B cell (C). Scale bar = 10um

Figure 2-6. Pericellular matrix synthesized by MM B cells includes HA: MM B cells express intracellular HA



PEA in combination with indirect HA staining was used to verify the existence of HA molecules in the pericellular matrix detected by PEA on Fig. 2. MM CD19$^+$ B cells were cultured for 48h and than were incubated with B-HABP. HA binding to B-HABP was visualized using streptavidin Alexa 594. (A) CD19$^+$B cell without HA staining. (B) The cell and pericellular matrix around MM CD19$^+$B cells which excluded fixed erythrocytes was stained with streptavidin Alexa 594 indicating the presence of HA in the pericellular matrix. (C) Merged image of PEA and HA staining. (D) The cells were treated with HAase which degraded the HA pericellular matrix. (E) HAase treatment also diminished cell surface and intracellular HA staining, which served as a negative control for B-HABP reagent. (F) Staining cells with B-HABP also detected intracellular HA in permeabilized MM CD19$^+$B cells. The staining pattern suggests that intracellular HA is distributed along the cell cytoskeleton and perinuclear compartment of MM B cells. Additionally, for one MM patient, weak nuclear staining of HA was observed in CD19$^+$B cells (not shown). Scale bar = 10 μm.

Chapter 2. 6. References

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Chapter 3: Predisposing polymorphisms and progressive accumulation of somatic mutations in the Hyaluronan Synthase 1 gene may lead to Waldenstrom's Macroglobulinemia.

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S.A.independently conceived the idea, designed and supervised all aspects of the research, performed the research, data analysis and interpretation of the results, wrote the manuscript; S.T. is director of Waldenstrom's Macroglobulinemia program at Dana-Farber Cancer Center, Boston, MA and provided patient samples; J.J.H., C.M. and E.S. provided technical help in cloning and sequencing experiments; L.M.L and M.J.M provided patient sampless; T.R. provided clinical contributions and suggestions while writing the manuscript; A.R.B. served as director of hematology clinic at the Cross Cancer Institute, grant genotyping experiments, provided clinical contributions and intellectual contributions for the manuscript; L.M.P. supervised S.A., contributed to research design and data interpretation.

Chapter 3. 1. Introduction

Waldenström's macroglobulinemia (WM), a B cell lymphoproliferative disorder, is characterized by monoclonal immunoglobulin M paraproteinemia and by the infiltration of lymphoplasmacytic cells into the bone marrow (BM). The incidence of WM is highest among Caucasians, with only 5% of all WM patients being of African descent, suggesting a role for genetic factors in the development of this disease. Although familial cases of WM occur in a minority of patients, molecular diagnostic and/or prognostic markers or predisposing elements for WM remain elusive[1,2].

In WM patients we have shown overexpression of the hyaluronan synthase 1 (HAS1) gene and detected aberrantly spliced transcripts of this gene in a majority of WM patients[3]. We previously demonstrated upregulation of the HAS1 intronic splice variant, HAS1Vb, in WM B cells[3]. Over-expression and/or aberrant expression of the HAS1 gene, which maps to the chromosomal location 19q14.3, has been reported in a variety of malignancies[4-12]. HAS1 has been implicated in malignant transformation[13]. Furthermore, in multiple myeloma, aberrant intronic splicing of HAS1 pre-mRNA correlates with significantly reduced survival[4]. The mechanisms involved are unknown, but the apparent synthesis of intracellular Hyaluronan (HA) by HAS1Vb may alter RHAMM-dependent mitotic events[14-16].

Pre-mRNA splicing, which plays a major role in the production of proteome complexity, is a very intricate process performed with the precision of one nucleotide. This process requires the activity of over 100 proteins and at least 5 small nuclear RNA-protein particles (snRNP)[17,18]. The sequence elements of the pre-mRNA that determine splicing specificity are 5' and 3' splice sites (SS), branch points (BP), and polypyrimidine tracts (PPT) of splicing[19,20]. These classical splicing elements are necessary but by no means sufficient for pre-mRNA splicing. The efficiency of this process is controlled by sequences located outside exon-intron boundaries, in the vicinity of splicing elements[19-21]. These splicing elements, exonic and intronic enhancers (ESE, ISE) and silencers (ISE, ISS) attract two families of protein, the serine/arginine (SR) family of proteins, splicing factors or co-factors, and the heterogeneous nuclear ribonucleoproteins (hnRNP)[22-26]. Both protein families play a significant role in splicing by modulating the strength of native and/or activating cryptic SS. Some of these proteins are involved in nuclear export of mature mRNA[27].

Even though this process is highly regulated by a complex repertoire of splicing factors with various splicing motifs, there is ample evidence for splicing defects in genes associated with susceptibility and/or progression of cancer[28-39]. It has been shown that splicing defects result from genetic variations (GVs) detected not only in the sequences of classical splicing elements but also within exons and introns[19,40-46]. These "deep" intronic GVs cause aberrant splicing of many disease-related genes by creating or strengthening cryptic SS and splicing elements[47-53].

In order to gain insight into the mechanism(s) underlying aberrant splicing of HAS1 in WM, we screened WM patients for the HAS1 833A/G SNP located upstream of aberrantly spliced exon 4 and for the existence of GVs in the vicinity of splicing elements. Our results suggest that the single nucleotide polymorphism (SNP) HAS1 833 A/G can induce aberrant splicing of HAS1 in the context of recurrent GVs detected in exons 3, 4 and intron 3, 4. Significantly, our analysis demonstrated that recurrent GVs are distributed as signature clusters of inherited germline origin GVs and acquired tumor and/or hematopoietic origin GVs, that occur in both coding and noncoding regions of the HAS1 gene that are involved in splicing. Inherited HAS1 GVs may predispose individuals to development of WM, and the acquisition of somatic HAS1 mutations in B cells may further promote oncogenic events in WM. In the context of an inherited predisposition that is detected in most WM patients, the acquired tumor and hematopoietic origin GVs may provide valuable markers for assessing the risk of transformation to WM.

Chapter 3. 2. Materials and Methods

Patients and Controls

The present study includes 91 patients with Waldenstrom's macroglobulinemia, the majority of which are clinically classified as being non-familial in nature. Patients were recruited independently at Cross Cancer Institute (Edmonton, Alberta), University of Alberta Hospital and Dana-Farber Cancer Institute (Boston, MA). All patients were diagnosed between 2000-2005 according to the consensus recommendations from the 2nd International Workshop on Waldenstrom's Macroglobulinemia[54,55]. Peripheral blood (PB), bone marrow (BM) and buccal samples were taken at the time of diagnosis or at follow up, after approval from the University of Alberta and the Alberta Cancer Board

Institutional Review Boards or the Dana-Farber Institutional Review Board, after informed consent. In addition, this study included a control group of 128 healthy donors (HDs) of both genders. This control group includes genomic DNA (gDNA) samples isolated from 100 PB and 28 buccal swab samples taken from the individuals residing in Alberta.

Tissue and sample preparation.

Tissue and sample preparation was conducted as previously described by Szczepek et al.[56]. Total RNA samples were isolated from sorted CD20$^+$ peripheral blood mononuclear cells (PBMC) or bone marrow (BM) cells (>96% purity) as described previously[4]. gDNA samples from patients and healthy donors were isolated using QIAamp DNA Blood mini kit (Qiagen) according to the manufacturer's instructions. Cryopreserved cells were never used.

RT-PCR, capillary electrophoresis and DNA fragment analysis

Expression of full length HAS1 (HAS1FL) and its splice variants was evaluated in WM patients and HDs, as previously described by Adamia et al. with final data analysis using GeneMapper software v 2.0 (Applied Biosystems, Foster City, CA)[4].

Genotyping

Samples were genotyped for the HAS1 833 A/G polymorphism with the Applied Biosystems TaqMan 5′ allelic discrimination assay-by-design (assay ID C_2184427_10. Applied Biosystems (ABI)). ABI prohibits disclosure of the primer sequences. However, the TaqMan MGB probe sequence was AGTCCAGAGGGTTAAGGATCCGCAC [A/G]TCCCCACCAACAGCCCCTACCCGGG. This assay includes allele-specific probes labeled with FAM or VIC. The FAM labeled probe detects allele "G", while the VIC labeled probe detects allele "A". Real-time allelic discrimination PCR was conducted according the manufacturer's instructions in a 25 or 12.5-μl reaction mixture containing 20 or 10ng of templates. Thermal cycling was performed on the PRISM™ABI7700 sequence detection system (ABI) under the following conditions: 95°C for 10min, and 40cycles of 92°C for 15sec and 60°C for 1min. Fluorescence was detected with the PRISM™ABI7700 Sequence Detector and genotypes were culled with the allele detection software incorporated into the instrument. This assay was tested and evaluated by sequencing of the gDNA samples of a small cohort of patients which were screened for HAS1 833A/G SNP. Both methods yielded identical results, validating the ABI allelic discrimination assay for screening the larger cohort of patients in our institution. A set of samples that had been tested by both methods were always used as a control to standardize each experiment. This approach allowed us to compare results obtained from the experiments performed at different times. Additionally, any given allelic discrimination experiment included negative controls for the reaction i.e. reaction without template. All WM samples were analyzed in the same laboratory using the same lot number for the assay reagents provided by ABI.

Cloning and sequencing

gDNA samples for cloning and sequencing were isolated from 5 WM patients, all of whom expressed HAS1 splice variants. The HAS1 gene segments were amplified using two approaches described in Figure 3-1, while the primer sets used for the amplification of the HAS1 segments are listed in Table 3-1. PCR conditions were as follows: 50ul PCR reaction mix contained 50ng gDNA, 5ul of 1XPCR buffer, 2mM MgSO4, 0.2mM dNTPs, 0.4mM HAS1 primer set (Table 3-1, Figure 3-1), and 0.5U High Fidelity (HiFi) Platinum Taq (Invitrogen). The PCR cycling parameters were: denaturation for 5min at 94°C, followed by denaturation for 30sec at 94°C, annealing for 40sec at 60^0C, and extension at 68^0C for 5min for 35 cycles, with a final extension period of 10min at 72^0C. The HAS1 PCR products were cloned into the pCR4 TOPO TA cloning system and sequenced as previously described[4]. After cloning of HAS1 gene segments in multiple cell types from each WM patient, at least 6 subclones from each cloned PCR product were sequenced, in both directions. Because of the significant overlap between primer sets, for some regions of HAS1 gene as many as 12-18 subclones were sequenced both directions. The analysis included 636 sequencing reactions.

Chapter 3. 3. Results

Expression of HAS1 and its variants in WM patients

Previous HAS1 gene profiling analyses conducted on 11 WM patient samples demonstrated upregulation of HAS1 and its splice variants transcripts, HAS1Va, HAS1Vb and HAS1Vc[3,28]. Additionally, single cell analysis of these transcripts in a limited number of patients showed that a majority of WM BM CD20$^+$B cells, 97-76%, express HAS1 variant transcripts[3]. 97% of WM BM CD20$^+$B cells expressed HAS1Va, while 76% of these cells expressed HAS1Vb. Expression of HAS1Vc transcripts were detected in 25% of the WM BM CD20$^+$B cells. However, HAS1FL transcripts were detectable only 3% of the cells. In addition, we screened HAS1 variant transcripts in PB CD20$^+$B cells from two of the WM patients for whom BM CD20$^+$B cells were analyzed for the expression of HAS1 splice variants. 68% of PB CD20$^+$B cells expressed HAS1Va transcripts, while 11% of these cells expressed HAS1Vb and 3% expressed HAS1Vc. For each patent we screened 100 cells for HAS1 variant profiling at single cell level. All expressed HAS3 transcripts confirming the integrity of the RNA. Our expanded cohort now includes BM CD20$^+$B cells obtained from BM aspirates of 4 patients, BM cells from 21 patients, and PBMC from 12 patients, a total of 37 cases. As shown by Table 2, a majority of patients express HAS1 variants alone or in combination with each other and HAS1 full length (HAS1-FL). Of 37 patients analyzed, 29 (78%) expressed HAS1Va, 12 (32%) of patients expressed HAS1Vb and 8 (22%) expressed HAS1Vc. We did not detect HAS1 splice variants in BM cells obtained from 3 healthy individuals (data not shown). Our analysis also demonstrated that 15/37 (41%) of patients express the intronic splice variants, HAS1Vb and HAS1Vc in WM BM and PB cells at comparable levels. These samples, screened for HAS1 gene profiling, were taken at time of diagnosis.

SNP HAS1 833 A/G Genotyping

It is well documented in the literature that splicing is affected by genetic mutations[19,29,41-51,57-61]. In a previously reported bioinformatic analysis of the HAS1 gene, we identified

HAS1 833A/G as a potential contributor to HAS1 aberrant splicing[28]. Using the pre-tested Taqman allelic discrimination assay for HAS1 833A/G we determined the genotypes of this SNP in 91 WM patients and 128 HDs. In addition, we screened sorted T cells from PBMC of 11 patients included in this cohort. HAS1 833A/G genotyping analysis demonstrated that the incidence of the HAS1 833G/G genotype was significantly increased in WM cases as compared with a control group of HDs (P=0.00031; Table 3-3). Among 91 WM cases analyzed thus far, 86.8% of patients were homozygous for the HAS1 833 "G" allele compared to 64% of HD controls. In WM patients we did not detect any individual homozygous for the HAS1 833 "A" allele; furthermore, only two healthy donors (1.6%) were homozygous for allele "A" (Table 3-3). Also, the HAS1 833A/G SNP allele frequencies observed were the same regardless of the type of samples analyzed; no differences were found between fractionated and unfractionated samples or from WM B and T cells with respect to a particular genotype. As shown in Table 3-3, overall allele frequencies for 833"G" and 833"A" are 93.4% and 6.6% respectively in WM patients, while in HDs the frequency of these alleles were 81.3% and 18.8% respectively. To evaluate the accuracy of the case-control study, we assessed Hardy-Weinberg Equilibrium in both, case and control groups for the HAS1 833 A/G SNP. No significant differences were found when the observed and expected allele frequencies were compared (Chi-square *P=0.79* for WM cases, *P=0.35* for HDs). Thus, the observed genotype and allele distribution frequency in both the case and control groups were consistent with Hardy-Weinberg equilibrium.

To evaluate whether the increased homozygosity for the HAS1 833A/G SNP detected in WM patients was related to aberrant splicing of the HAS1 gene, we compared the expression patterns of HAS1 and its variants with the HAS1 833A/G SNP genotypes of the patients. We found that increased homozygosity for the HAS1 833"G" allele detected in WM patients (locus Ch19q13.4) correlated with expression of intronic splice variants of HAS1 (HAS1Vb and HAS1Vc), while patients expressing HAS1Va and/or HAS1-FL were either heterozygous or homozygous for HAS1 833 allele"G" (Table 3-4). Although, all patients expressing HAS1 intronic splice variants were homozygous for HAS1 833 allele"G", not all patients with the G/G genotype expressed the intronic splice variants. Thus, the HAS1 833G/G genotype appears to be necessary but not sufficient for aberrant splicing of HAS1.

Sequencing analysis of exons and introns 3 and 4 of HAS1

To identify additional GVs which may promote aberrant splicing of HAS1, we selected 5 WM patients expressing all splice variants of HAS1 transcripts and sequenced exons (3 and 4) and introns (3 and 4) that surround alternatively spliced HAS1 exon 4. Additionally, in this study, we included 2 HDs. gDNA samples for HAS1 exon/intron sequencing were obtained from buccal epithelial cells, CD20$^+$B and CD3$^+$T cells from BM of patient #1, CD20$^+$B and CD3$^+$T cells from BM of patient #2, unfractionated BM cells, CD20$^+$B (PBMC) and buccal epithelial cells from patient #3, and unfractionated BM cells from patients #4 and #5. For HDs, CD20$^+$B and CD3$^+$T cells from PBMC were analyzed. At least 6 subclones were sequenced for each region of the HAS1 gene for each subset of cells. This approach enabled us to evaluate frequencies of the HAS1 GVs within a given patient and subsequently, distinguish whether the patient was heterozygous (e.g. 2-3/6 subclones had the alternative nucleotide, mutated or wild type) or homozygous (5-6/6 subclones had the alternative nucleotide, mutated or wild type) for a given GV.

Sequencing analysis revealed 98 unique or recurrent GVs in the HAS1 gene that include substitutions, insertions and deletions. 76 of these GVs are unique (detected in only 1/5 patients) to individual WM patients in this limited cohort of 5 patients, and 22 of them are recurrent, defined as occurring in 2 or more of the 5 patients analyzed (Supplemenary Table 3-1; Table 3-5). These GVs were mapped against the HAS1 gene sequence reported in the NCBI database and against the HAS1 exons and introns sequenced from B and T cells of HDs as part of this study. Next, GVs were classified according to their occurrence in various cell populations obtained from WM patients (Table 3-5; Supplement Table 3-1).

Overall classification of GVs detected in WM patients

1) GVs detected only in malignant WM B cells were classified as **tumor specific**. These GVs were absent from autologous T and buccal cells, and were not detected in cells from healthy donors. 2) GVs identified in lymphocytes, including both B (malignant) and T cells (non-malignant) were conditionally classified as being of **hematopoietic origin**. Although present in B and T cells of patients, these HAS1 GVs were absent from the buccal epithelial cells of patients, which indicates that these are somatic GVs restricted to WM lymphocytes and are most likely present in other hematopoietic cells of WM

patients. They were also absent from B and T cells of HDs. 3) GVs identified in buccal cells, T cells and B cells were classified as **germline origin** GVs. Their presence in all cell types tested from a given patient, particularly buccal cells, implies that they are inherited mutations. Among these inherited germline origin GVs, we identified GVs classified as novel SNPs, which have been detected in three or more patients and are absent from the NCBI database. In WM patients, we detected increased homozygosity for the novel SNPs. In 6/6 subclones sequenced, we found a polymorphic nucleotide (mutated) in the position of the HAS1 DNA, where a given novel SNP was mapped. In contrast, both HDs sequenced here were heterozygous for these "novel SNPs". We also identified a high frequency of the polymorphic alleles (mutated) of 10 SNPs previously reported in the NCBI database, defined as present in 3-5 of the 5 WM patients analyzed. Because the cohort used for sequencing was small, those GVs defined as "unique" may gain some degree of recurrence when a larger cohort is analyzed.

GVs detected in exons 3 and 4 (Table 3-5; Figure 3-2; Supplement Table 3-1).

We detected 23 and 3 coding mutations in WM HAS1 exon 3 and 4, respectively. One recurrent tumor specific mutation, a missense a>T (CH 56912068), led to an amino acid change, Tyr to Phe, in exon 3 of HAS1. Additionally, in exon 3, we detected 22 GVs unique to a given WM patient. These 22 GVs included 12 tumor specific (4 silent and 8 missense), 3 hematopoietic (1 silent and 2 missense) and 7 germline origin (3 silent and 4 missense) GVs. In exon 4 we detected 3 GVs out of which one is a unique tumor specific mutation and two are recurrent missense mutations classified as novel SNPs, with predicted amino acid substitutions of Met/Leu and Arg/Pro.

GVs detected in introns 3 and 4 (Table 3-5; Figure 3-2; Supplement Table 3-1). In intron 3 of the HAS1 gene, we identified clusters of 5 recurrent and 15 unique GVs. The 5 recurrent GVs include one that is tumor specific, one of germline origin and three NCBI-SNPs. The 15 unique mutations include 11 that are tumor specific, two hematopoietic and two germline origin mutations. We detected increased homozygosity for mutated alleles of the 3 NCBI-SNPs in all 5 WM patients.

In intron 4, we identified 13 recurrent and 37 unique GVs. Among the 13 recurrent GVs were two tumor specific, one hematopoietic origin, three germline origin, one novel SNP, and six NCBI-SNPs. The 37 unique GVs found in intron 4 included 25 tumor specific, seven hematopoietic and 5 germline origin mutations (Table 3-5).

Most importantly, in WM patients we identified 3 distinct clusters of recurrent GVs through sequencing of HAS1 minigenes (as in Figure 3-1b) from WM patients (Table 3-5), all of which include NCBI-SNPs identified in introns 3 and 4, plus germline origin and tumor specific insertions and one deletion (Figure 3-2). These germline origin and tumor specific insertions and one deletion were detected within the specific sequence stretch of intron 4 (1st"T" stretch, 2nd"T" stretch and TTTA repeats). These designated sequences are located in the vicinity of splicing elements at the 3' end of intron 4, where the partial intron retention takes place. For simplicity, NCBI-SNPs shared by all sets of the GVs and GVs detected within the 1st"T" stretch, 2nd"T" stretch and TTTA repeats of intron 4 all together will be referred to as "shared GVs". These shared GVs appeared to cluster with additional recurrent HAS1 GVs. The first GV cluster includes the "shared

GVs" plus a recurrent tumor specific missense mutation a>T (CH6912068) detected in exon 3. The second GV cluster is comprised of the "shared GVs" and three additional novel SNPs, of which one was identified in intron 4 (CH56910041) and two in exon 4; these are missense mutations a>T (CH56911346) and g>C (CH56911348) in exon 4 that lead to amino acid changes Met to Leu and Arg to Pro, respectively. Additionally, this second cluster of GVs includes one recurrent tumor specific transition (CH5611668). The third GV cluster includes "shared GVs" and three NCBI-SNPs (rs4802849and, rs4802848, rs4802850) that were absent from the HDs sequenced here. All 5 patients analyzed were homozygous for mutated alleles of NCBI-SNPs included in the third GV cluster.

Recurrent HAS1 GVs in WM promote HAS1 aberrant splicing.

We explored possible effects of recurrent sets of GVs on HAS1 gene splicing. Using web based bioinformatics tools (the ASD database, EMBL-EBI Alternative splicing workbench, and ESE finder) we predicted and evaluated classical (BP and PPT) and cis-splicing elements (ESE, ISE, ESS, ISS) of the wild type (WT) and mutated (MT) HAS1 alleles. Details of these analyses will be reported elsewhere (Adamia et al. in preparation).

Briefly, bioinformatic analysis demonstrated significant changes between WT and MT exons 3 and 4, with respect to accumulation of splicing factors and co-factors (SR and hnRNP proteins). These analyses showed that the WT or MT exon 3 has a higher affinity to accumulate SR proteins, as compared to aberrantly spliced out exon 4. These SR proteins are crucial for the selection of 5'SS and 3'SS and splicesosome stabilization on the BP[22,32,62]. No differences were found between WT and MT exon 3 with respect to the accumulation of hnRNPs which bind mainly ESS and ISS and promote exon exclusion. Interestingly, in MT exon 4, as compared to WT, the analysis predicted a massive accumulation of hnRNPs across the entire exon 4. The binding of these proteins at several sites of an exon could silence any given exon[63-66]. No difference was found between WT and MT exon 4 with respect to SR protein distribution. Thus, as a result of GVs detected in WM, HAS1mutated exon 4 has a greatly increased susceptibility to aberrant splicing, and mutated exon 3 attracts the proteins that activate distal SS and most likely promotes exclusion of exon 4.

During the *in silico* prediction analysis of introns 3 and 4, we focused on the regions of the 5' and 3'ends of these introns. We did not detect any significant differences between WT and MT intron 3 with respect to SRs or hnRNP motif distribution. However, analysis of intron 4 demonstrated accumulation of a significant number of SR and hnRNP binding motifs in mutated intron 4. Additionally, the existence of alternative PPTs and BPs were predicted in MT intron 4, conferring a high ability to accumulate U2SF65 protein, which stabilizes the spliceosomal complex necessary for the first stage of the splicing reaction[22,25,32,62,67].

An evaluation of WT and MT intron 3 and 4 with respect to classical and *cis*-splicing elements suggests that retention of intron 4 in HAS1Vb and HAS1Vc transcripts most likely is supported by the activation of cryptic splicing elements resulting from recurrent GVs identified in WM patients.

Chapter 3. 4. Discussion

Sequencing analysis of HAS1 gene segments obtained from WM patients revealed 98 GVs in exons 3, 4 and introns 3, 4 of HAS1, many of which occur in pre-mRNA splicing elements. These include both inherited germline origin and acquired somatic HAS1 GVs. The HAS1 exons 3, 4 and introns 3, 4 of WM patients exhibit a homogeneous distribution of inherited polymorphisms, those reported in NCBI as well as novel SNPs that may predispose individuals to WM. For the HAS1 833A/G SNP, the majority of WM patients were homozygous for the "G" allele, the frequency of which is significantly higher in WM patients than in HDs. In addition, the sequencing analysis also revealed a sequential accumulation of somatic GVs in HAS1 gene. These GVs were of two types, those present in both nonmalignant T cells and malignant B cells, but absent from buccal cells, and a second set that was restricted to the tumor cells and absent from both non-malignant T cells and buccal epithelial cells. We speculate that inherited polymorphisms and mutations most likely predispose individuals to WM, while somatically acquired hematopoietic origin GVs in concert with tumor cell specific GVs may represent progressive genetic changes that lead to oncogenesis and WM. Unexpectedly, a substantial proportion (22/98) of these GVs were recurrent, defined as occurring in 2 or more of WM patients. The recurrence of the same somatically acquired GVs in the multiple unrelated individuals whose gene segments were sequenced suggests that they must have been strongly selected during oncogenesis. Bioinformatic analysis predicts that these HAS1 GVs promote aberrant splicing of HAS1 by changing sequence motifs necessary for the binding of splicing factors and co-factors and by activating alternative, cryptic classical splice sites.

This work confirms the expression of intronic HAS1 splice variants in patients with WM[3]. The intronic splice variants of HAS1, HAS1Vb and HAS1Vc, are expressed in one third of WM patients, and 86% of WM patients overall express at least in one of the HAS1 splice variants. The inherited and somatic recurrent HAS1 GVs that characterize WM patients may contribute to this aberrant splicing of HAS1, since they occur in the vicinity of HAS1 splicing elements.

The mechanism(s) generating aberrant intronic HAS1 splice variants in WM patients remain speculative. However, a growing body of evidence suggests that the effects on splicing can be predicted from a gDNA sequence through identification of inherited or somatic GVs altering highly conserved classical splicing signals and/or modifying cis-splicing elements[37,40,42,47,48,50-52,57,68]. 10% of the characterized mutations reported in the "Human Gene Mutation Database" occur at exon-intron boundaries; these mutations contribute to cancer and other genetic diseases (The Human Gene Mutation Database. www.hgmd.cf.ac.uk/ac/index.php.) However, the number of mutations in this database is an underestimate, since this database does not include "deep" intronic GVs, which modulate splicing elements located deep within the introns. Thus, to understand the cause of the aberrant splicing of HAS1 in WM patients, we screened HAS1 exons/introns in the NCBI database for reported mutations. We determined the allelic frequency of the HAS1 833 A/G SNP in WM patients, since this SNP is located in exon 3, upstream of aberrantly spliced exon 4. In addition, the HAS1 833A/G SNP abrogates the binding motif of the SF2/ASF splicing factor as reported previously by us[28]. The genotyping analysis of 91 WM patients and 128 HDs demonstrated that WM patients showed increased homozygosity for the 833"G" allele as compared to HDs (*P=0.00031*). However, 62% of screened HDs were also homozygous for allele "G", indicating that this genetic change by itself does not lead to WM. Nevertheless, correlation analysis between HAS1 variant expression and genotyping demonstrated that HAS1Vb and HAS1Vc are found only in patients homozygous for HAS1 833"G" allele. In contrast, HAS1Va is found in both 833A/G and G/G WM patients. We speculate that homozygosity of allele "G" at position 833 is necessary for aberrant splicing of HAS1, but is by no means sufficient to activate the cryptic SS that lead to aberrant exclusion of exon 4 and/or partial retention of intron 4 and the production of HAS1 splice variants. Thus, additional GVs must drive aberrant splicing of HAS1 in WM patients.

Our hypothesis was tested in five WM patients expressing all three HAS1 splice variants. We sequenced the segments of the HAS1 gene which participate in the relevant splicing reactions. In WM patients, we identified 22 recurrent and 76 unique GVs in exons 3 and 4, and introns 3 and 4 of HAS1 (Supplement Table 3-1). Given the small cohort used for sequencing, the possibility exists that these "unique" GVs will have some degree of recurrence when evaluated in a larger cohort. Interestingly, most of the patients were homozygous for the somatically acquired, recurrent, tumor specific GVs. In addition to the implications for oncogenesis in WM, these recurrent tumor specific GVs may prove valuable as "universal" molecular signatures for WM, by providing a diagnostic and monitoring biomarker for the disease.

The categories of germline and hematopoietic origin recurrent HAS1 GVs, in addition to their conceptual interest as a progressive accumulation of GVs leading to cancer, also have practical value. Germline origin GVs were detected at high frequency in WM patients, and were present in both malignant B cells and non-malignant T and in buccal epithelial cells. The majority were not detected in HDs or reported in the NCBI database. Their presence in buccal epithelial cells implies that they were inherited and may predispose to development of WM. Additionally, we identified 3 germline origin GVs, probable novel SNPs, for which HDs analyzed were heterozygous, while 3/5 WM patients were homozygous. Thus, individuals who are homozygous for mutated alleles of these novel SNPs may have a genetic predisposition for WM. This is clearly shown in a much larger cohort of WM patients tested for the NCBI-SNP, HAS1 833A/G, demonstrating significantly increased homozygosity in WM patients as compared to HDs. This suggests that HAS1 833G/G, and likely the other homozygous inherited mutations identified here, are predisposing elements for WM, and clinically valuable identifiers of individuals at risk of developing WM.

We also identified a set of GVs that were found in malignant B and non-malignant T cells of WM patients but were absent from buccal epithelial cells, indicating an acquired somatic mutation that must have occurred early in hematopoietic development, leading to their presence in both nonmalignant T cells and malignant B cells. These GVs were absent from B and T cells of HDs that were analyzed. The existence of the recurrent hematopoietic origin HAS1 GVs in WM patients suggests that they may represent the initial stages of a HAS1 mutational cascade that culminates in a malignant transformation to WM within the B lineage. They also provide useful clinical biomarkers for monitoring the transition from pre-malignant to malignant disease.

Similar to other levels of gene control, the regulation of splicing involves *cis* and *trans* elements[18-20,25,27,33,39,41,69]. *Cis*-elements are sequence motifs located across exons and introns, while *trans*-elements are proteins, splicing factors, and co-factors that bind to *cis*-elements (ESE, ISE, ISE, ISS) and promote the splicing. ESEs are required for exon inclusion and are binding sites for members of the SR family of proteins, while ESS and ISS attract an extended family of hnRNPs and silence segments of DNA. Our sequence analysis demonstrated that the occurrence of recurrent and/or patient specific GVs is non random. In WM, these GVs are clustered, forming mutational hot spots in the vicinity of splicing elements, ESE, ISE, ISE, ISS. Using web based bioinformatics tools, we evaluated the effects of clusters of recurrent GVs identified in WM patients on HAS1 gene splicing. *In silico* comparison of splicesomal assembly between wild type and GVs in HAS1 gene gave a pattern that precisely predicts partial retention of intron 4 and aberrant splicing of the HAS1 gene. Individuals who acquire these mutational clusters appear to be at high risk of developing WM, likely based on the influence of HAS1 mutational clusters on normal HAS1 splicing patterns and the consequent impact of aberrantly spliced HAS1 on the oncogenic process leading to WM.

This study shows for the first time that germline origin GVs in the HAS1 gene may predispose individuals to WM and that a progressive accumulation of recurrent somatic GVs in the tumor cells themselves accompanies development of WM. This suggests that HAS1 plays a central role in the oncogenic process and that clinical monitoring of patients to determine the mutational patterns of their HAS1 genes may provide a predictive test for monitoring the transition to overt malignancy. In addition, the pattern of tumor-specific recurrent somatic mutations provides a common clonal marker for all WM patients, making feasible the regular monitoring of each patient to unequivocally identify clinically cryptic tumor cells, as well as precise molecular monitoring of the response to treatment.

Table 3-1. Nucleotide sequences of primer sets used in the study

HAS1 gene segments	Primer sequences
Exon 3	Reverse: GGGGTCTGTGCTGATCCTGG Forward: GCTTCCAGTTTTATCCCATC
Intron 3	Reverese: CTTCCACTGTGTATCCTGCATC Forward: AACTGCTGCAAGAGGTTATTCC
Exon 4	Reverse: TGGGGTTGGAACTGGAGATG Forward: CATGCACACACGCTAGGATA
Intron 4a	Reverse: GCTCAGCATGGGTTATGCTA Forward: GTATCCCCGCAGCTTAAACA
Intron 4b	Reverse: TTGGGATAATCCAGGGGAAT Forward: CAAGATGGGTGTGGTTGCTA
Intron 4c	Reverse: GGTAGCAACCACACCCATCT Forward: AGGAATGAGGGCATCATCG
Exon 5	Reverse: CTCGCCCCCGTGCAGGTACA Forward: AGGCCCCCAAGCAGCAGCAGCGC
HAS1 minigene	Reverse: CTTCCACCTTACAGGTCTGTGACT Forward: CCACTCTGGTTCATGGTGACTA

Primers were designed using the "Primer 3" or "Gene Tool" programs based on the published sequence of the HAS gene. The mfold bioinformatics tool was used to identify secondary structures of the segments of HAS1 gene to be amplified by these primers. This enabled us to evaluate accessibility of the template for the primer set.

Table 3-2. Expression of HAS1 and its variants in WM patients

Sample type	Patients	HAS1Va	HAS1Vb	HAS1Vc	HAS1
2BM, 1PB (CD20$^+$)	#1-3	-	-	-	+
7BM, 2PB	#4-12	+	-	-	-
5BM (CD20$^+$), 3PB	#13-20	+	-	-	+
BM	#21	+	+	-	-
1BM, 2PB	#22-24	+	+	-	+
BM	#25-26	+	+	+	-
BM	#27-29	+	+	+	+
BM	#30	+	-	+	-
1BM, 1PB (CD20$^+$)	#31-32	+	-	+	+
2BM, 1PB (CD20$^+$)	#33-35	-	+	-	-
2BM	#36-37	-	-	-	-
WM total	N=37	78%	32%	22%	51%

Profiling of HAS1 and its variants was performed using PBMC or BM cells. Additionally, this analysis includes HAS1 and its variant transcripts obtained from $CD20^{+}B$ cells. These cells were sorted from PBMC (PB $CD20^{+}$ B) or BM aspirates (BM $CD20^{+}B$) of patients with WM. Expression of HAS3 transcripts, which was detected in every sample tested, served as a positive control to validate the RT-PCR reaction (data not shown).The first part of the Table reports the expression patterns of HAS1 and variants in individual patients tested, while the second part summarizes the gene profiling results. This demonstrates that splicing events take place in 32/37 (86%) of analyzed patients, among whom 47% express intronic splice variants. Primers included in the reactions were validated by their ability to amplify transcripts isolated from the CCL 110 cell line, which expresses HAS1 and HAS1Va transcripts. The primer sequences and reaction conditions used for this study were as previously described.[2]

Table 3-3. HAS1 833 A/G genotyping in WM patients

WM cases

	Genotype distribution observed				Genotype distribution expected	
	Numbers	Frequency	Frequency %		Frequency	Frequency %
n of patinets	91	1	100		1	100
GG	79	0.868	86.813	p^2	0.872	87.248
AG	12	0.132	13.187	2pq	0.123	12.317
AA	0	0	0	q^2	0.004	0.435
n of alleles	182		100			
G (p)	170	0.934	93.407			
A(q)	12	0.066	6.593			

*P=0.0003*1

Healthy donors

	Genotype distribution observed				Genotype distribution expected	
	Numbers	Frequency	Frequency %		Frequency	Frequency %
n of patients	128	1	100		1	100
GG	82	0.641	64.063	p^2	0.66	66.016
AG	44	0.344	34.375	2pq	0.305	30.469
AA	2	0.016	1.563	p^2	0.035	3.516
n of alleles	256		100			
G (p)	208	0.813	81.250			
A(q)	48	0.188	18.750			

This cohort of 91 cases includes 14 fractionated (BM CD20$^+$B cells) and 27 unfractionated BM samples, plus 20 fractionated (PB CD20$^+$B cells) and 30 unfractionated PBMC samples. Statistical significance of increased homozygosity for the HAS1 833G/G genotype in WM versus HDs was determined using Two-sided Fisher's exact or chi-square test (*p=0.00031*, Chi-square=11, OR=0.31, 95 % CI=0.16-0.62). The observed distributions of HAS1 833 A/G SNP genotypes (GG, AA, or AG) in WM patients and HDs were matched to the expected values, as predicted by the Hardy-Weinberg Equilibrium.

Table 3-4. HAS1 and novel variant transcripts expression versus HAS1 833 A/G genotype

Number of patients	HAS1 and its variant expression profiles	Genotype
n=3	*HAS1*	A/G or G/G
n=9	*HAS1Va*	A/G or G/G
n=8	*HAS1Va, HAS1*	A/G or G/G
n=1	*HAS1Va*, HAS1Vb	G/G
n=3	HAS1Vb	G/G
n=3	*HAS1Va*, HAS1Vb, *HAS1*	G/G
n=1	*HAS1Va*, HAS1Vc	G/G
n=2	*HAS1Va*, HAS1Vb, HAS1Vc	G/G
n=2	*HAS1Va*, HAS1Vc, *HAS1*	G/G
n=3	*HAS1Va*, HAS1Vb, HAS1Vc, *HAS1*	G/G

This table demonstrates the correlation analysis between expression patterns of HAS1 novel variants and HAS1 833 A/G genotyping. For this analysis WM patients were grouped based on HAS1 and variant transcripts expression patterns and next, compared with HAS1 833A/G SNP genotyping results. As Table 4 demonstrates, WM patients expressing HAS1 intronic variants, HAS1Vb and HAS1Vc (both are underlined) are exclusively homozygous for HAS1 833"G" allele. Gene expression profiling was carried out in parallel to the genotyping experiments. The RNA and gDNA for expression analysis and genotyping, respectively, were isolated from the same sample of a given patient using the RNA/DNA Trizol isolation method.

Supplement Table 3-1. GVs detected in WM patients

Exon 3

1	2	3	4	5	6	7
Transition	g>A	56912041	Unique	Tumor specific	Gly>Gly	Silent
Transition	t>C	56912051	Unique	Germline origin	Cys>Arg	Missense
Transversions	t>A	56912051	Unique	Tumor specific	Cys>Ser	Missense
Transversions	t>C	56912051	Unique	Tumor specific	Cys>Ser	Missense
Transition	t>C	56912056	Unique	Germline origin	Val>Ala	Silent
Transition	t>C	56912058	Unique	Germline origin	Cys>Cys	Silent
Transversions	a>T	56912068	Recurrent	Tumor specific	Tyr>Phe	Missense
Transition	g>A	56912077	Unique	Hematopoietic origin	Cys>Tyr	Missense
Transition	t>C	56912078	Unique	Germline origin	Cys>Arg	Missense
Transition	t>C	56912079	Unique	Hematopoietic origin	Ala>Ala	Silent
Transition	c>T	56912080	Unique	Germline origin	Ala>Val	Missense
Transition	a>G	56912092	Unique	Tumor specific	Asn>Ser	Missense
Transition	t>C	56912125	Unique	Tumor specific	Phe>Ser	Missense
Transition	c>T	56912144	Unique	Tumor specific	Leu>Leu	Silent
Transition	t>C	56912145	Unique	Tumor specific	Pro>Pro	Silent
Transversions	t>G	56912169	Unique	Tumor specific	Gly>Gly	Silent
Transversions	t>A	56912182	Unique	Tumor specific	Val>Glu	Missense
Transversions	t>A	56912203	Unique	Tumor specific	Val>Glu	Missense
Transition	g>A	56912210	Unique	Germline origin	Val>Ile	Missense
Transition	g>A	56912217	Unique	Germline origin	Leu>Leu	Silent
Transversions	g>C	56912238	Unique	Tumor specific	Arg>Ser	Missense
Transition	a>G	56912240	Unique	Tumor specific	Arg>Gly	Missense
Transition	t>C	56912255	Unique	Hematopoietic origin	Cys>Arg	Missense

Intron 3

1	2	3	4	5	6	7
Transition	c>T	56911477	Unique	Germline origin		
Transition	a>G	56911493	Unique	Germline origin		
Transition	t>C	56911500	Unique	Hematopoietic origin		
Transition	t>C	56911513	Unique	Tumor specific		
Transition	g>A	56911526	Recurrent	Germline origin		
Transition	a>G	56911562	Unique	Hematopoietic origin		
Transition	t>C	56911599	Unique	Tumor specific		
Transversions	a>C	56911637	Unique	Tumor specific		
Transition	a>G	56911668	Recurrent	Tumor specific		
Transition	t>C	56911734	Unique	Tumor specific		
Transversions	t>A	56911750	Recurrent	SNP-NCBIrs11669079		
Transition	a>G	56911759	Unique	Tumor specific		
Transition	t>C	56911762	Unique	Tumor specific		
Transversions	g>T	56911776	Unique	Tumor specific		
Transversions	t>G	56911816	Unique	Tumor specific		
Transition	g>A	56911831	Recurrent	SNP-NCBI rs 11084109		
Transition	t>C	56911858	Unique	Tumor specific		
Transition	g>A	56911889	Recurrent	SNP-NCBI rs 11084110		
Transition	g>A	56911966	Unique	Tumor specific		
Transition	a>G	56911977	Unique	Tumor specific		

Exon 4

1	2	3	4	5	6	7
Transversions	a>T	56911346	Recurrent	novel SNP	Met>Leu	Missense
Transversions	g>C	56911348	Recurrent	novel SNP	Arg>Pro	Missense
Transition	c>t	56911386	Unique	Tumor specific	Thr>Thr	Silent

Intron 4

1	2	3	4	5	6	7
Transition	a>G	56910835	Unique	Tumor specific		
Transition	c>T	56909199	Unique	Hematopoietic origin		
Transition	c>T	56909217	Unique	Germline origin		
Transition	t>C	56909252	Recurrent	Tumor specific		
Transition	g>A	56909253	Unique	Hematopoietic origin		
Transition	c>T	56909262	Unique	Tumor specific		
Transversion	t>G	56909417	Unique	Tumor specific		
Transition	t>C	56909482	Unique	Tumor specific		
Transition	g>A	56909487	Unique	Germline origin		
Transition	a>G	56909495	Unique	Hematopoietic origin		
Transition	t>C	56909521	Unique	Tumor specific		
Deletion	del C	56909573	Unique	Tumor specific		
Insertion	(T)s	56909589	Recurrent	Germline origin		
Deletion	del T	56909589	Recurrent	Tumor specific		
Transition	t>C	56909635	Unique	Tumor specific		
Deletion	CC	56909663	Unique	Hematopoietic origin		
Transition	t>C	56909746	Unique	Tumor specific		
Transversion	t>A	56909757	Recurrent	SNP-NCBI rs8103845		
Insertion	(T)	56909761	Recurrent	Germline origin		
Transition	c>T	56909763	Recurrent	SNP-NCBI rs8104157		
Transition	a>G	56909830	Unique	Tumor specific		
Transition	c>T	56909852	Unique	Tumor specific		
Transition	a>G	56909896	Unique	Tumor specific		
Transition	a>G	56909899	Unique	Tumor specific		
Transition	t>C	56909989	Unique	Tumor specific		
Transversions	a>T	56910030	Unique	Tumor specific		
Transversions	g>t	56910041	Recurrent	Novel SNP		
Transition	c>T	56910083	Unique	Hematopoietic origin		
Transition	a>G	56910128	Unique	Germline origin		
Transversions	c>G	56910154	Recurrent	SNP-NCBI rs4802848		
Transversions	c>A	56910155	Recurrent	SNP-NCBI rs4802849		
Deletion	del G	56910345	Unique	Tumor specific		
Transversions	g>T	56910447	Unique	Germline origin		
Transversions	g>C	56910493	Recurrent	SNP-NCBI rs4802850		
Transition	a>G	56910538	Unique	Tumor specific		
Transition	a>G	56910661	Unique	Tumor specific		
Transition	g>A	56910711	Recurrent	SNP-NCBI rs7254072		
Transversions	t>G	56910738	Recurrent	SNP-NCBIrs 11667949		
Transversions	g>C	56910770	Unique	SNP-NCBI rs 11667974		
Transition	a>G	56910811	Unique	Germline origin		
Transition	t>C	56910815	Unique	Tumor specific		
Transition	t>C	56910856	Unique	Tumor specific		
Transition	g>A	56910864	Unique	Hematopoietic origin		
Transition	a>G	56910929	Unique	Tumor specific		

1	2	3	4	5	6	7
Transition	a>G	56911098	Unique	Tumor specific		
Transition	a>G	56911122	Unique	Tumor specific		
Transition	c>T	56911149	Unique	Tumor specific		
Transition	a>G	56911224	Unique	Hematopoietic origin		
Transition	t>C	56911245	Unique	Tumor specific		
Transition	g>a	56911283	Unique	Tumor specific		
Insertion	x TTTA	56909436-35	Recurrent	Germline origin		
Deletion	xTTTA	56909439-36	Recurrent	Hematopoietic origin		

1- Types of genetic variations.
2- Nucleotide changes.
3- Changed nucleotide position in the chromosome 19q13.4.
4- Occurrence frequency of genetic variations among the patients analyzed.
5- Classification of genetic variations based on their occurrence in cell types.
6- Effects on the protein.
7- Translationally silent or missense genetic variations

Table 3-5. Recurrent GVs detected in WM patients

Exon 3

1	2	3	4	5	6
Transversions	a>T	56912068	Tumor specific	Tyr>Phe	cluster #1

Intron 3

1	2	3	4	5	6
Transition	g>A	56911526	Germline origin		
Transition	a>G	56911668	Tumor specific		cluster #2
Transversions	t>A	56911750	SNP-NCBI rs 11669079		shared GVs
Transition	g>A	56911831	SNP-NCBI rs 11084109		shared GVs
Transition	g>A	56911889	SNP-NCBI rs 11084110		shared GVs

Exon 4

1	2	3	4	5	6
Transversions	a>T	56911346	novel SNP	Met>Leu	cluster #2
Transversions	g>C	56911348	novel SNP	Arg>Pro	cluster #2

Intron 4

1	2	3	4	5	6
Transition	t>C	56909252	Tumor specific		cluster #3
Insertion	(T)s	56909589	Germline origin		shared GVs
Deletion	del T	56909589	Tumor specific		shared GVs
Transversion	t>A	56909757	SNP-NCBI rs 8103845		
Insertion	(T)s	56909761	Germline origin		shared GVs
Transition	c>T	56909763	SNP-NCBI rs 8104157		shared GVs
Transversions	g>t	56910041	Novel SNP		cluster #2
Transversions	c>G	56910154	SNP-NCBI rs 4802848		cluster #3
Transversions	c>A	56910155	SNP-NCBI rs 4802849		cluster #3
Transversions	g>C	56910493	SNP-NCBI rs 4802850		cluster #3
Transition	g>A	56910711	SNP-NCBI rs 7254072		shared GVs
Transversions	t>G	56910738	SNP-NCBI rs 11667949		shared GVs
Insertion	x TTTA	56909436-435	Germline origin		shared GVs
Deletion	xTTTA	56909439-436	Hematopoietic origin		shared GVs

1- Types of genetic variations.
2- Nucleotide changes.
3- Changed nucleotide position in the chromosome 19q13.4.
4- Classification of genetic variations based on their occurrence in cell types.
5- Effects on the protein.
6- Translationally silent or missense genetic variations.

Figure 3-1. Two strategies for sequencing HAS1 gene segments

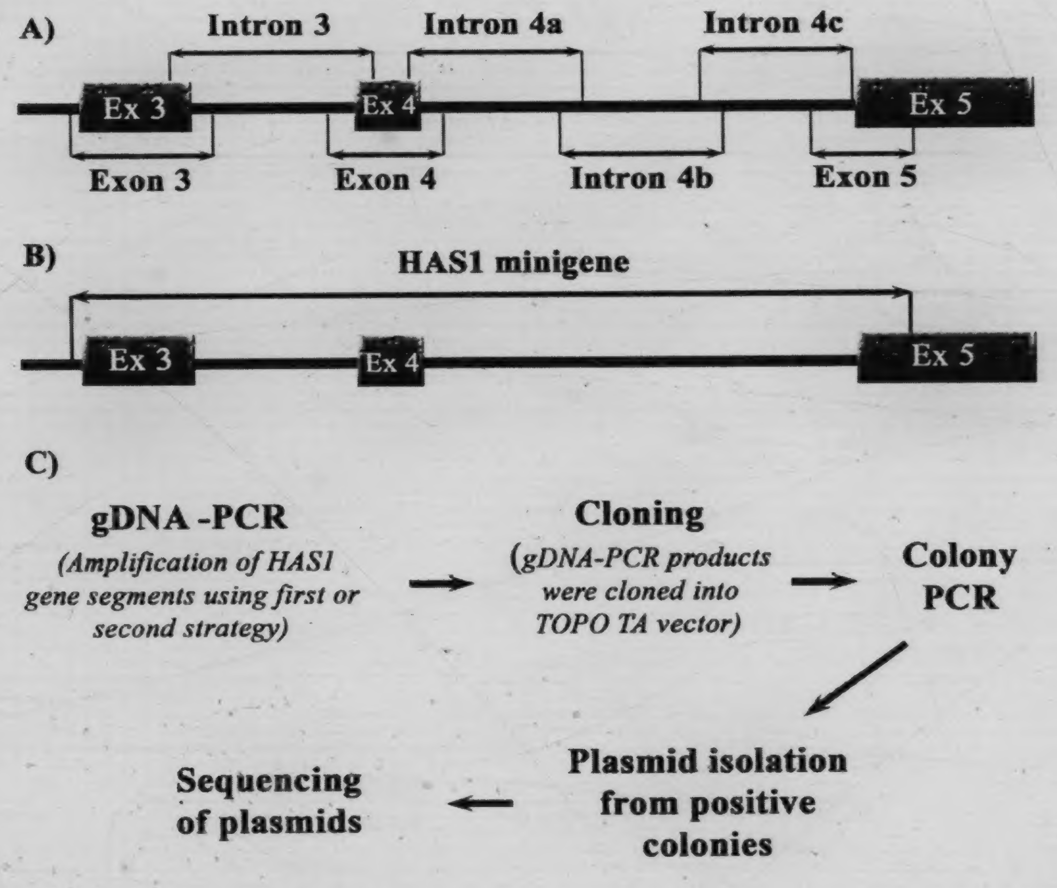
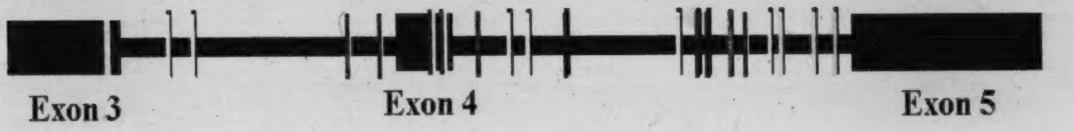

A)
Intron 3
Intron 4a
Intron 4c
Ex 3
Ex 4
Ex 5
Exon 3
Exon 4
Intron 4b
Exon 5
B)
HAS1 minigene
Ex 3
Ex 4
Ex 5
C)
gDNA -PCR
(Amplification of HAS1 gene segments using first or second strategy)
Cloning
(gDNA-PCR products were cloned into TOPO TA vector)
Colony PCR
Plasmid isolation from positive colonies
Sequencing of plasmids

Figure 3-1 A) describes the first strategy employed for amplifying the HAS1 gene segments. Overlapping reverse and forward primers were designed against exons and introns of HAS1 gene to identify any genetic variation that contributes to the HAS1 splicing in WM patients. B) describes the second strategy used to determine whether the recurrent GVs are present in context as a set of clusters. C) Flow chart of cloning and sequencing of HAS1 gene segments of WM patients and HDs. gDNA PCR and cloning was carried out as described in methods. Next, we picked 24 to 48 subclones to screen and identify the existence of inserts (gDNA PCR product of HAS1 gene segment) in the TOPO TA plasmid using appropriate primer sets for each segments. While using the first strategy of amplifying HAS1 gene segments from the patients or HDs, we cloned 6 segments of the HAS1 gene. For each segment, 6 positive subclones were selected and a total of 36 plasmids for each patient were isolated and sequenced both directions using M13 and T7 sequencing primers as described previously.[2]. For each patient, as many as 72 sequencing reactions were analyzed to identify genetic variations in the HAS1 gene segments indicated. Using the second strategy, after cloning we obtained 30 HAS1 minigene plasmids. Each plasmid was sequenced using overlapping HAS1 gene specific primers, the localizations of which are shown on Figure 1A. From the second strategy, we obtained 180 sequencing reactions. Because we used overlapping primers either in gDNA PCR (first strategy) or for sequencing (second strategy) we analyzed 50-60 sequencing reactions for some segments of HAS1 gene. The HAS1 gene segments of two HDs (B and T cells) were sequenced using the first strategy. The genetic variations identified in WM patients were assessed based on a total of 636 sequencing reactions.

Figure 3-2. Relative distribution of recurrent GVs



A schematic representation of distributions of recurrent GVs (vertical lines) detected in the HAS1 gene of WM patients. Black boxes represent exons while introns are shown with solid lines.

Chapter 3. 5. References

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Chapter 4: Single nucleotide polymorphisms (SNPs) and recurrent mutations in the Hyaluronan Synthase 1 (HAS1) gene may predispose to Multiple Myeloma (MM) and contribute to disease oncogenesis

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S.A. independently conceived the idea, designed and supervised all aspects of the research, performed the research, data analysis and interpretation of the results, wrote the manuscript; J.H. assisted in cloning and sequencing experiments; PPP designed figures for the manuscript; M.J.M. providedpatient samples and reviewed the manuscript; T.R. provided clinical contributions and suggestions while writing the manuscript; A.R.B. served as director of hematology clinic at the Cross Cancer Institute, grant genotyping experiments, provided clinical contributions and intellectual contributions to the manuscript; L.M.P. supervised and designed research and assisted in data interpretation.

Chapter 4. 1. Introduction

Multiple Myeloma (MM) is a B cell malignancy characterized by the accumulation of plasma cells (PCs) in bone marrow (BM), the existence of lytic bone lesions, and the presence of monoclonal protein in serum and urine. The median survival of MM patints from diagnosis is prolonged to 3-4 years with conventional therapy[1,2]. High-dose therapy with stem cell rescue appears to extend the disease-free and overall survival of patients by up to 4-5 years[3,4]. However, this treatment approach cures few, if any; thus, the disease remains incurable. The panels of biochemical markers used to diagnose and monitor MM include the proliferative activity of PCs and levels of β_2-microglobulin. Recently, clinically predictive cytogenetic markers have been identified[5]. However, the outcome of MM patients is highly heterogeneous, with considerable variability in biomarkers among individuals. The question arises whether these diagnostic markers are surrogate and reflect disease activity rather than the biology of the tumor. This may compromise the ability to make fully informed decisions regarding diagnosis and treatment. The molecular mechanism(s) underlying disease initiation and progression remain unclear and no markers for disease predisposition are as yet available.

The Hyaluronan synthase (HAS) gene, which maps into chromosomal location 19q13.4, is a plasma membrane protein that produces the extracellular matrix molecule hyaluronan (HA)[6-8]. HA plays a significant role in the progression of many types of cancer[9-17]. Three human isoenzymes of HAS have been identified in humans, HAS1, HAS2 and HAS3[7,18]. Previously, we identified aberrant splice variants of the HAS1 gene, termed HAS1Va, HAS1Vb and HAS1Vc, in patients with MM and Waldenstrom's macroglobulinemia (WM)[12,13]. HAS1Va is a result of alternative exclusion of exon 4, while HAS1Vb and HAS1Vc are intronic splice variants of HAS1, which are expressed exclusively by circulating compartments of the MM clone. Our previous statistical analysis in MM patients showed that the expression of HAS1Vb strongly correlates with poor survival.[13] In WM, gene expression analysis conducted at the single cell level demonstrated upregulation of HAS1 intronic splice variants in malignant WM B cells[12]. In WM patients, we identified inherited and, somatic genetic variations (GVs) in the vicinity of splicing elements that are associated with HAS1 gene splicing (Adamia et al., submitted). The mechanism(s) whereby HAS1 aberrant splicing leads to reduced survival in MM is unknown, but may involve abnormalities caused by HAS1Vb synthesis of intracellular HA[19-21].

It is easy to imagine how missense mutations, which lead to amino acid changes in any given protein, and nonsense mutations, which promote a premature termination of translation, could cause disease. However, genetic variations (GVs) detected within non-coding regions of genes (e.g. introns) in the vicinity of splicing elements, specifically exonic and intronic enhancers (ESE and ISE), and suppressors (ESS and ISS) are underemphasized. As previously reported, we detected clusters of recurrent and unique GVs in non-coding and coding segments of the HAS1 gene from different cell types of patients with WM (Adamia et al., submitted). In the current study, we report the existence of recurrent and unique GVs in the HAS1 gene of MM patients. Similar to WM patients, these HAS1 GVs are either inherited, (i.e. of germline origin) or acquired (i.e. somatic), some of which are present at high frequency in non-malignant cells from MM patients. In these patients, we identified HAS1 GVs specific to MM malignant PCs and circulating B cells, as well as somatic HAS1 GVs specific to hematopoietic progenitor cells (HPCs) purified from G-CSF mobilized blood autografts of MM patients. These HAS1 GVs, which conditionally are termed as "hematopoietic origin", were identified in hematopoietic progenitor and T cells as well as MM tumor cells. Our results suggest that germline origin HAS1 GVs detected in MM patients may predispose individuals to MM, providing a biomarker to predict the risk of overt MM. Hematopoietic origin somatic HAS1 GVs that accumulate before and during MM oncogenesis predict a higher level of risk. A combination of hematopoietic origin and tumor specific HAS1 GVs can be used as prognostic biomarkers to identify clinically cryptic malignant cells prior to diagnosis of overt MM, to monitor patients at risk of progressive disease or to monitor response to treatment and emerging relapse. The impact of hematopoietic origin GVs on HAS1 gene splicing is manifest only in the context of accompanying tumor-specific HAS1 GVs that in combination give rise to the clinically significant aberrant splicing of HAS1. Bioinformatic analyses presented here indicate that recurrent clusters of GVs detected in the HAS1 gene contribute to aberrant splicing of HAS1 pre-mRNA, ultimately leading to aberrant HAS1 protein, malignant progression and reduced survival of MM patients.

Chapter 4. 2. Materials and Methods

Patients and Controls

This study was approved by the University of Alberta and Alberta Cancer Board research ethics committees. After informed consent, peripheral blood mononuclear cells (PBMC) were obtained from 270 MM patients. The patients involved in the study were diagnosed according to the standard criteria between 1994 and 2005[22]. Total RNA isolated from the CCL 100 cell line was used as a control in gene profiling experiments. Blood samples (100) or buccal swabs (28) from 128 anonymous healthy controls were obtained from laboratory staff and patients presenting at the emergency room of the University of Alberta Hospital with conditions unrelated to malignancy.

Sample purification

Peripheral blood (PB), BM and buccal epithelial cells (BECs) from the patients were taken at the time of diagnosis or at follow up. PB and BM cells were purified on Ficoll-Hypaque Plus (Amersham-Pharmacia Biotech, Uppsala, Sweden) density gradients using standard conditions. Total RNA samples for gene expression analysis were isolated from 5 to 10 million cells suspended in Trizol Reagent (Invitrogen, Carlsbad, CA). PB $CD19^{+}$B cells, BM $CD38^{hi}CD138^{+}$PC, PB $CD3^{+}$T, and from $CD34^{+}45^{low}$HPCs from fresh G-CSF mobilized blood autografts of MM patints were selected as previously described[23,24]. Genomic DNA (gDNA) from unfractionated and/or sorted cells was isolated as previously described (Adamia et al. submitted) (Chapter 3). Cryopreserved cells were never used.

RT-PCR capillary electrophoresis and DNA fragment analysis

Profiling of HAS1 and its variant transcripts was performed using PBMC from 172 MM patients, as previously described[13].

Genotyping

270 samples from patients with MM were genotyped for the HAS1 833A/G SNP as previously described (Adamia at al, submitted). In this study 128 healthy donor (HD) samples of both genders were used as a control to evaluate the significance of allele frequencies of the HAS1 833A/G SNP carried out in MM patients. For genotyping analysis the control samples were collected from the individuals residing in the same geographical area as patients to avoid the potential bias of population stratification. Calling of genotypes for MM and HDs was blinded. Details of the assay verification have been previously reported (Adamia et al., submitted (Chapter 3)). The statistical significance of HAS1 833A/G genotype distribution in MM patients was determined using a chi-square test and exact test for independence proportion. Odds ratio and their 95% confidence intervals were also calculated.

Statistical evaluation

The clinical parameters of the patients for survival analysis were collected as previously described.[13] In this current study we correlated the expression of HAS1 and aberrant splice variants with the survival days of the patients. Survival distributions were determined by the Kaplan-Meier method using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California, USA). Statistical significance for the test was set at a $P=0.05$ using two-sided analysis. Odds ratios and 95% confidence intervals were also calculated.

Cloning and sequencing

Cloning and sequencing of HAS1 gene segments were performed on gDNA isolated from multiple sorted cell subsets obtained from six MM patients. Patients known to express HAS1 and all of its splice variants were specifically chosen for these experiments. The HAS1 gene segments were amplified, cloned and sequenced using two strategies as previously described (Adamia et al., submitted (Chapter 3)). Thus, using the first strategy, 500-800bp segments of exons and intons of the HAS1 gene were amplified. The reverse and forward primers used in these PCR reactions overlapped significantly, which enabled us to evaluate the accuracy of PCR and sequencing reactions. Next, during the cloning, up to seven positive sub clones were selected and plasmids with inserts of interest were generated. These plasmids were sequenced in both directions. Since each insert length was 500-800bp we were able to obtain complete sequences of the plasmids in both directions. Using our first strategy of sequencing we obtained up to 60 sequencing reactions for each sample, which covered the vicinity of exon-intron bounderes of the HAS1 gene. Thus, this approach enabled us to evaluate if a given patient was heterozygous or homozygous for the mutations identified in the HAS1 gene segments. However, using a second strategy we generated up to 60 minigenes of the HAS1. Each minigene was sequenced in both directions by the HAS1 gene specific primer sets used in the first strategy of HAS1 sequencing. A second strategy of HAS1 gene sequencing enabled us to determine whether the recurrent GVs detected in patients were present as a cluster of GVs. The regions of HAS1 that were sequenced included exons 3 and 4 and introns 3 and 4 and part of exon 5. For MM patients #1 and #2 gDNA samples were obtained from purified BM CD138$^+$PC, circulating B and T cells (PB CD19$^+$B and CD3$^+$T) from PBMC, purified CD34$^+$45^{low}HPCs from mobilized blood, and BECs. For patients #3 and #4, we selected B and T cells from PBMC, while from patients #5 and #6 gDNA samples were isolated from PBMCs. Up to seven subclones were sequenced both directions for each region of the HAS1 gene for each subset of the cells. Thus, up to 670 sequencing reactions were performed and analyzed to determine patterns of GVs of HAS1 gene in MM patients.

Bioinformatic analysis

The classical and *cis*-splicing elements of HAS1 gene were assessed using web-based Bioinformatics tools. Bioinformatics analyses were preformed on wild type (WT) or mutated (MT) exons (3, 4) and introns (3, 4) of the HAS1 gene using “ESE finder” web interface (Release 2.0). The sequence-motif matrices that predict functional ESEs recognized by the SR proteins SF2/ASF, SRp40, SRp55, and SC35 are derived from the pool of functional enhancer sequences tested in vivo and in vitro systems. Additionally, cis-splicing elements (exonic and intronic enhancers (ESE, ISE) and suppressors (ISE, ISS), splicing branch point (BP) and polypyrimidine tract (PPT) of WT and MT HAS1 gene segments were mapped and evaluated using the publicly available Alternative Splicing Database (ASD) and EMBL-EBI Alternative splicing workbench. The scores of splicing BP were calculated using Wrapper tool-intron analysis software. The prediction analyses were carried out using default values, which were adjusted for background nucleotide composition.

Chapter 4. 3. Results

Expression of HAS1Vb correlates with increased lytic bone lesions

A previous cohort of MM patients in which expression of HAS1Vb intronic variant transcripts were correlated with poor patient outcome included 58 individuals[13]. In the work reported here, we analyzed a much larger cohort of MM patients. HAS1 gene expression profiling for 172 MM patients demonstrated that HAS1 splice variants are expressed either alone, or in combination with each other and/or with HAS1 full length (HAS1FL). Of the 172 patients analyzed, 122 (71%) expressed HAS1Va, 106 (62%) expressed HAS1Vb, 35 (20%) expressed HAS1Vc, and 119 (69%) expressed HAS1FL. The majority of MM patients, 117/172 (68%), expressed HAS1 intronic variants HAS1Vb and HAS1Vc alone or in combination with each other and/or HAS1FL.

Of 172 patients analyzed for HAS1 variant profiling, the survival data for correlation analysis was available for 144 patients. Statistical analysis of samples taken from 146 untreated MM patients shows that expression of HAS1Vb transcripts correlates with poor survival of patients with a significance of *P=0.005* (Figure 4-1A). Statistical analysis suggested that median survival for MM patients expressing HAS1Vb transcripts is 583 days. Thus, expression of HAS1Vb remains characteristic of patients with aggressive disease. Next, we compared the survival of patients with lytic bone lesions with or without accompanying expression of HAS1 variants. Among 80 MM patients with lytic bone disease, the 44 patients that expressed HAS1Vb had statistically shorter survival than those patients lacking expression of HAS1Vb transcripts *(p=0.02)* (Figure 1B). Median survival of patients with lytic bone disease expressing HAS1Vb transcripts was 498 days, while median survival of patients with lytic bone disease but lacking HAS1Vb transcripts was 842 days.

SNP HAS1 833 A/G Genotyping

Since expression of intronic HAS1Vb variant transcripts correlated with reduced survival, we investigated the influence of HAS1 833A/G genotype on HAS1 pre-mRNA splicing. A growing body of evidence suggest that splicing can be effected by somatic or inherited GVs including single nucleotide polymorphisms (SNPs), through altering highly conserved classical splicing signals BP, PPT, splicing sites (SS)) and *cis*-splicing elements (ESE, ISE, ESS, ISS)[25-33]. A bioinformatic analysis conducted on the HAS1 gene suggested that HAS1833A/G may promote aberrant splicing of HAS1 likely in the context of other GVs[34]. This hypothesis was first verified in WM patients (Adamia et al., submitted). A significant majority of WM patients that were screened for the HAS1833A/G SNP showed increased homozygosity for allele "G" as compared to HDs.

Using the same pre-tested Taqman allelic discrimination assay for HAS1833A/G SNP, we screened 270 MM patients. This HAS1833A/G genotyping analysis of MM patients demonstrated that the frequency of 833G/G genotype was significantly increased in MM cases *(P=0.000002;* Table 1) as compared to a control group of 128 HDs (from Adamia et al. submitted; (Chapter 3)). Among 270 MM cases analyzed thus far, 230 of 270 (85.1%) of patients were homozygous for the HAS1 833"G" allele compared to 64 % of HDs. In the group of MM patients analyzed, we detected only 4 of 270 (1.5%) individuals homozygous for the HAS1 833"A" allele (Table 4-1). Similar to MM patients, among 128 HDs we detected 2 (1.6%) individuals homozygous for allele "A" (Adamia et. al. submitted; (Chapter 3)). In addition, we calculated overall allelic distributions of HAS1 833 alleles "A" and "G" in MM patients (Table 4-1). This analysis showed that overall allele frequency for 833"G" was 92%, while allele frequency for 833"A" was 8.2%. The frequency of these alleles in HDs was 81.3% and 18.8% respectively. Using the Hardy –Weinberg proportion, we calculated the expected frequencies of "A" and "G" alleles and corresponding genotypes within the case and control groups (Table 4-1). Next, using a chi-square test, we determined whether observed genotype frequencies in MM patients and HDs were consistent with Hardy-Weinberg equilibrium (HWE). No deviations from HWE were found in MM patients or HDs. The chi-squared P values for MM patients and HDs were 0.199 and 0.35, respectively. This assessment of HWE provided a check to ensure that genotyping errors or population stratification do not explain observed results in MM patients with respect to increased homozygosity for allele 833"G".

Sequencing analysis of exons and introns 3 and 4 of HAS1

Since sequencing of the HAS1 gene segments from WM patients identified both unique and recurrent HAS1 GVs, we asked whether there exist GVs which are specific to MM patients and are absent from HDs and WM patients. We also evaluated the distribution patterns of GVs detected in HAS1 gene exons and introns shared by MM and WM patients. To address this, we selected 6 MM patients with aggressive disease expressing all splice variants of the HAS1 gene. We sequenced exons 3 and 4 and introns 3 and 4, chosen because these segments of the HAS1 gene are hotspots for splicing aberrations in MM and WM (Adamia et al. submitted; (Chapter 3)). gDNA samples for HAS1 exon/intron sequencing were obtained from MM BECs, PBMCs, purified subsets of PC, B and T cells, and CD34+45^{low}HPCs (from freshly obtained mobilized blood). For two of the MM patients, HAS1 gene segments from all five cell subsets were sequenced.

HAS1 sequencing analysis performed in MM patients revealed 108 unique and recurrent GVs in the HAS1 gene, predominantly in introns 3 and 4 (Supplement Table 1). These GVs include 99 substitutions, four insertions, and five deletions. Within this small cohort of 6 MM patients, 81 of the HAS1 GVs were unique to individual MM patients and 27 were recurrent in three to all six of the MM patients (Supplement Table 4-1; Table 4-2). To characterize the patterns of GVs identified in MM patients, we used our previous classification (Adamia et al., submitted; (Chapter 3)). Briefly, GVs detected only in MM PCs and circulating B cells, but absent from non-malignant T cells and from BECs, were classified as **tumor specific** (Figure 4-2). GVs found in both malignant and non-malignant MM cells, including MM PCs, circulating B and T cells, and

CD34^{+}45^{low}HPCs from mobilized blood but absent from autologous BECs, were conditionally classified as **hematopoietic origin** (Figure 4-2). The GVs detected in all analyzed cell types including BECs were classified as **germline origin** GVs (Figure 2). Germline origin GVs, which we identified in more than one patient and which are not reported in the NCB database, were classified as **novel SNPs.**

GVs identified in exon 3 and 4 (Supplement Table 4-1; Table 4-2).

GVs identified in the HAS1 gene of MM patients were mapped against the HAS1 sequence reported in the NCBI database and against the sequences of HAS1 exons and introns obtained from WM patients and HDs previously reported (Adamia et al., submitted; (Chapter 3)). In exon 3, this analysis identified 5 recurrent (one tumor specific, two hematopoietic and one germline origin) and 11 unique GVs. Two of these recurrent GVs are silent and three are missense mutations that lead to amino acid changes from Cys>Arg, Val>Ala, and Cys>Ser. In exon 4 we identified three GVs, one tumor specific and two hematopoietic origin. However, when sequences of the HAS1 gene segments from MM were mapped against the sequences from WM, we identified recurrent HAS1 GVs which were shared between MM and WM patients (Table 4-3). Hereafter, these GVs will be referred as "overlapping GVs". In MM exon 3 we identified 8 overlapping GVs. These GVs included four HAS1 exon 3 mutations (2 missense and 2 silent) that were apparently unique when only MM patients were considered, but which were clearly recurrent based on their presence in WM patients. The other 4 mutations of exon 3 were recurrent in MM patients as well as in WM patients (Table 4-3). This analysis also identified two of the three previously MM unique GVs in exon 4 as overlapping, hematopoietic origin GVs in MM and WM. All overlapping GVs which had provisionally been identified as unique to either MM or WM patients were reclassified in Table 4-3 as recurrent GVs based on their recurrence within the larger group of 11 patients (6MM and 5WM). We anticipate that many of the HAS1 GVs provisionally identified as unique will be shown to have some degree of recurrence when evaluated in a much larger patient cohort (in progress).

GVs identified in introns 3 and 4 (Supplement Table 4-1; Table 4-2).

Sequencing analysis of the HAS1 introns 3 and 4 from MM patients identified 22 recurrent and 67 unique GVs. Among the four recurrent GVs detected in MM intron 3, one was of hematopoietic origin and three were SNPs reported in the NCBI database (NCBI-SNPs). However, we identified 18 recurrent GVs in MM intron 4; these GVs included one tumor specific, eight germline origin and two hematopoietic origin GVs, plus seven NCBI-SNPs. When sequences of the HAS1 introns 3 and 4 from MM and WM patients were compared, we identified overlapping GVs, three of which were present in intron 3, and 12 of which were distributed across intron 4 (Table 4-3). All three overlapping GVs identified in intron 3 of MM and WM were recurrent NCBI-SNPs. However, three unique GVs in intron 4, two tumor and one hematopoietic origin, were also present in WM patients and thus, these GVs were reclassified as recurrent. Other overlapping GVs of intron 4 were recurrent and included three germline origin GVs and six NCBI-SNPs. All overlapping GVs of intron 3 and 4 have been reclassified and become recurrent among MM and WM patients. If any given GV was present in about half of the subclones, it was evaluated as heterozygous at that allelic position, otherwise GV was identified as homozygous.

After classification of all recurrent GVs in MM patients that were similar in WM patients, we identified 3 clusters of recurrent GVs (Table 4-2). The clusters of the GVs identified in MM patients were compared to the clusters of GVs identified in WM patients. For simplicity, clusters of GVs detected in MM and WM patients will be referred to as "MM clusters" or "WM clusters", respectively. This analysis demonstrates that the shared GVs identified in WM clusters of GVs were also present in all clusters of GVs of MM patients. These shared GVs are distributed in the vicinity of splicing elements across the intron 3 and within the specific sequence stretch of intron 4, 1st "T" stretch, 2nd "T" stretch and TTTA repeats (Adamia et al. submitted; (Chapter 3)). These designated sequences of intron 4 are located in the vicinity of classical splicing signals at the 3' end of the intron. The sets of MM clusters of GVs #1, #2, and #3 are reported in Table 4-2. Interestingly, GVs comprising MM GV cluster #3 are identical with the WM GV cluster #3 with one exception: the MM cluster #3 includes a germline origin mutation located in MM exon 3 (CH56912051) instead of a mutation in intron 4 at location CH56909252 (in WM cluster #3). Two other HAS1 gene GV clusters from MM patients included GVs specific to MM patients. Thus, there appears to be considerable sharing of germline and hematopoietic orgin GVs between HAS1 genes of MM and WM. However, we also identified mutational events that are restricted to only MM or only WM. Surprisingly, even the group of tumor specific HAS1 GVs includes some that are shared between MM and WM, suggesting a fundamentally similar process of oncogenesis may precede both of these incurable cancers.

In silico analysis indicates that clustered GVs in HAS1 lead to aberrant splicing of HAS1 pre-mRNA

Recurrent GVs detected in the HAS1 gene of WM patients were able to modify *cis*-splicing elements such as ESE, ESS, ISE, and ISS and promote aberrant splicing of the HAS1 gene (Adamia et al., submitted; (Chapter 3)). In this study we investigated whether the overlapping GVs and clusters of GVs identified in MM patients predict the aberrant HAS1 splicing events that lead to HAS1Vb and the associated reduction in survival of MM patients. Our sequence analysis demonstrated that the occurrence of recurrent and/or patient specific GVs are non random. Using ESE finder and ASD workbench bioinformatics tools; we evaluated distribution of splicing elements in HAS1 exons 3, 4 and introns 3, 4 of WT and MT sequences. We also evaluated the affinity of splicing factors for the splicing elements of interest in exons 3 and 4. Recurrent HAS1 mutations in exon 3 caused rearrangement of motifs that increased affinity of MT exon 3 for the binding of splicing factors and co-factors which comprise the family of SR (Ser/Arg rich) proteins (SF2/ASF, SC35, SRp40, SRp55). Recruitment of these proteins could alter selection of 5' and or 3' splicing sites (Figure 4-3A,B)

No differences were found between WT and MT exon 3 with respect to the accumulation of hnRNPs (heterogeneous nuclear ribonucleoproteins) which bind mainly splicing suppressors and promote exon exclusion. However, GVs in exon 4 introduced new motifs that recruit hnRNP proteins which silenced exon 4 through competing and compromising bindings of the splicing factors and co-factors on this exon (Figure 4-4A,B).

In the analysis of introns 3 and 4, we did not detect any significant differences between WT and MT intron 3 with respect to SRs or hnRNPs specific motif distribution. Also, no difference was found when BP and PPT were mapped on WT and MT intron 3. However, it is important to note that because of its nucleotide composition, the PPT of intron 3 is weak as compared the alternative PPTs detected in MT intron 4.

Interestingly, prediction analysis showed that insertions and deletions in intron 4 in the context of other recurrent overlapping clusters of GVs strengthened a cryptic PPT and activated an alternative BP in this intron (Figure 4-4D). These clusters of GVs also masked the native 3' SS in intron 4 and activated the alternative upstream 3'SS of this intron (Figure 4-4D). Furthermore, accumulation of significant numbers of U2AF65/35 molecules on the alternative PPT and BP of MT intron 4, as compared with the native elements, predicts for more stable spliceosomal assembly on alternative BP and PPT (Figure 4-4D,E). The analyzed cluster of HAS1 GVs precisely predicts splicing events that are required for generation of HAS1Vb. This in silico analysis provides plausible and testable predictions of the mechanisms whereby recurrent clusters of GVs occurring in exons 3, 4 and introns 3, 4 lead to the partial retention of intron 4 and production of the HAS1Vb intronic splice variant in MM patients. Tests of these predictions are in progress.

Chapter 4. 4. Discussion

In this chapter we report the existence of inherited and somatic GVs in the HAS1 gene from MM. These GVs are classified as tumor specific, hematopoietic origin and gemline origin (Figure 4-2). Some of these GVs are detected in malignant and non-malignant cells from MM patients, suggestive of a progressive accumulation of GVs in HAS1 that lead to increasingly higher risk of developing overt MM. The biological relevance of these HAS1 GVs is emphasized by the fact that aberrant splicing of HAS1 pre-mRNA in MM correlates with reduced survival[13], and that the HAS1 GVs described here have been shown by *in silico* analysis to profoundly alter HAS1 splicing. This work may provide a mechanism for the known occurrence of aberrant HAS1 splicing in MM cells.

As confirmed here, aberrant splicing of the HAS1 gene occurs at high frequency in a group of MM patients with aggressive disease. HAS1 splice variant gene profiling analysis demonstrated that MM patients with bone lytic disease expressing a HAS1Vb intronic variant have significantly reduced survival compared to patients from the same group which were negative for HAS1Vb expression (*P=0.02*). HASVb transcripts may be valuable biomarkers to identify MM patients with aggressive disease and a poor prognosis.

Sequencing analysis conducted in MM patients, demonstrated a specific distribution of inherited and acquired GVs identified in the vicinity of *cis*-splicing elements. Moreover, we predicted that these GVs alter classical splicing signals of the HAS1 gene and consequently modify the patterns of HAS1 gene splicing. Thus, it is reasonable to classify these GVs as splicing mutations, although some of these GVs were shared between MM and WM suggesting some degree of similarities between these diseases.

However, most of the GVs detected in MM PC and B cells, but absent from BECs and non-malignant HPCs and T cells, were specific to MM and were absent from the malignant WM B cells. These tumor specific GVs were also absent from HDs whose HAS1 gene segments were previously sequenced and they are not reported in the NCBI database. Thus, recurrent GVs which are specific to MM malignant clones can be used as markers to identify malignant clones in MM patients at the time of diagnosis or to monitor patients during treatment. Also, the existence of GVs shared only between MM PC and circulating MM B cells supports the concept that MM PCs are derived from malignant B cells[13,23,35-40]. Since tumor specific HAS1 GVs are associated with HAS1Vb splicing as predicted by *in silico* analysis, and since expression of HAS1Vb transcripts are frequent in patients with aggressive disease as identified by statistical analysis, it is reasonable to speculate that tumor specific GVs can be used to identify MM patients in advanced clinical stages and with progressive disease. They may also provide valuable biomarkers for detecting clinically cryptic stages of overt MM and early stages of impending relapse.

We also identified somatic GVs which were exclusively detected in hematopoietic cells (PC, PB B and T cells) as well as in purified CD34^{+}45^{low}HPCs of MM patients. Their presence in HPS implies that they occur at the early stages of hematopoietic development and possibly are present in other hematopoietic cells which have not yet been tested.

Accumulation of somatically acquired HAS1 GVs in CD34^{+}45^{low}HPCs, in the context of inherited predispositions to MM, may represent a very early stage of oncogenesis, introducing HAS1 GVs. These GVs will be passed on to B lineage cells, and together with tumor specific GVs that are acquired exclusively in B cells and their plasma cell progeny, ultimately lead to overt MM.

As previous studies suggest, expression of HAS1Vb transcripts is characteristic of MM cells and has not been detected in other hematopoietic lineage cells such as T cells from MM patients. Thus, the HAS1 GVs which accumulate in CD34^{+}45^{low} HPCs, are necessary but by no means sufficient for oncogenesis and HAS1 gene splicing. The effects of these hematopoietic cell lineage-specific GVs on HAS1 splicing are manifested in malignant MM cells in the context of additional tumor specific HAS1 GVs, which are acquired by circulating B cells and passed to their plasma cell progeny. Finally, it is intriguing to speculate that, similar to leukemias, initiation events that contribute to MM pathogenesis may arise from GVs which first accumulate during the non-malignant or pre-malignant stages of hematopoietic differentiation[41-43].

In MM patients we also identified germline origin HAS1 GVs, two of which can be classified as potential novel SNPs, since these GVs were identified in more than two patients analyzed. Interestingly, we detected increased homozygosity for the mutated allele of these novel SNPs in all types of cells (PC, B, T, and BECs) from MM patients. One of the potential novel SNP (CH56912051) is also present in WM patients (Chapter 3). Additionally, we identified known NCBI-SNPs in MM patients that, similar to the potential novel SNPs in MM patients, showed increased homozygosity for the mutated allele. All these SNPs except two (rs4802850, rs11667949) also were identified in WM patients. Additionally, genotyping analysis of HAS1833A/G NCBI-SNP showed significantly increased homozygosity for allele "G" in MM patients (P=0.000002). It appears increased homozyosity for some HAS1 alleles in patients are characteristics of MM and WM patients.

Sequencing analysis also revealed the existence of overlapping GVs between MM and WM patients. Even though MM and WM are different diseases, both are B cell disorders and both express aberrantly spliced transcripts of HAS1. Additionally, of 27 overlapping GVs detected in MM patients, the majority are either hematopoietic or of germline origin. This evidence, among other findings reported in this manuscript, suggests that GVs which are associated with HAS1 aberrant splicing undergo mutational selection events, and leave a mutational "trace" throughout the hematopoietic cell lineage, including tumor cells. Thus, some of these GVs can be used as signatures to identify "at risk" individuals, while others provide valuable markers for diagnoses and monitoring of MM or WM patients.

Bioinformatic analysis indicates that the recurrent GVs detected in the HAS1 exons and introns from MM patients alter key splicing elements, the sequence motifs for binding of splicing factors and co-factors. The HAS1 GVs described here have been shown in silico to activate cryptic PPT and BP for splicing, thus creating a new splice site in precisely the position required for the splicing events that produced HAS1Vb transcripts in vivo

(Figure 4-4). This provides evidence that the HAS1 exonic and intronic GVs in splicing sites are clinically relevant and can be viewed as valid candidate biomarkers of disease. More extensive analysis of large patient cohorts and experimental evaluation of HAS1 GVs and splicing events are in progress.

Taken together, the three types of HAS1 GVs provide a powerful strategy for stratification of individuals at risk of MM. Those individuals with germline HAS1 polymorphisms have some degree of risk for MM. However, those individuals who in addition to inheriting HAS1 germline polymorphisms have also acquired HAS1 mutations, may be viewed as having a greatly increased risk of developing MM (or WM), and may merit closer monitoring. Finally those individuals who have detectable tumor specific HAS1 GVs could be viewed as having the greatest risk of MM and may perhaps merit preventive treatment should this become available. Furthermore, the early detection of tumor specific HAS1 GVs may identify early stage MM as it arises, prior to the appearance of clinically detectable disease.

Table 4-1. HAS1 833A/G genotyping in MM patients indicates significantly increased G/G homozygosity

MM cases

	Genotype distribution observed				Genotype distribution expected	
	Numbers	Frequency	Frequency %		Frequency	Frequency %
n of patients	270	1	100		1	100
GG	230	0.85	85.1	p^2	0.84	84.4
AG	36	0.13	13.3	2pq	0.15	14.96
AA	4	0.015	1.48	p^2	0.006	0.66
n of alleles	540		100			
G (p)	496	0.92	91.85			
A(q)	44	0.081	8.15			

Healthy donors

	Genotype distribution observed				Genotype distribution expected	
	Numbers	Frequency	Frequency %		Frequency	Frequency %
n of patients	128	1	100		1	100
GG	82	0.641	64.063	p^2	0.66	66.016
AG	44	0.344	34.375	2pq	0.305	30.469
AA	2	0.016	1.563	p^2	0.035	3.516
n of alleles	256		100			
G (p)	208	0.813	81.250			
A(q)	48	0.188	18.750			

This MM cohort of 270 patients included samples isolated from BM PC, PB CD19$^+$B and CD3$^+$T cells obtained from 7 patients. No differences were found between fractionated and unfractionated samples or among MM B, PC and T cells when HAS1 833A/G SNP genotypes were determined, indicating that the same HAS1 833 genotype was shared by both host and tumor. The increased homozygosity for allele "G" as compared to 128 HDs was determined using Chi-square test (P=0.000002, Chi-square=16, OR=0.40, 95%CI=0.25-0.63). No deviation from Hardy-Weinberg Equilibrium was found between the observed and expected distributions of HAS1 833 A/G SNP genotypes in MM patients and HDs. HDs were genotyped previously in chapter 3.

Table 4-2. Recurrent genetic variations are detected in HAS1 gene segments of MM patients

		Exon 3				
1	2	3	4	5	6	7
t>C	56912079	Recurrent	Hematopoietic origin	Ala>Ala	Silent	
t>C	56912056	Recurrent	Hematopoietic origin	Val>Ala	Missense	set #1
t>C	56912051	Recurrent	Novel SNP	Cys>Arg	Missense	set #3
t>A	56912051	Recurrent	Tumor specific	Cys>Ser	Missense	
		Intron 3				
1	2	3	4	5	6	7
t>A	56911750	Recurrent	NCBI rs 11669079			shared
g>A	56911831	Recurrent	NCBI rs 11084109			shared
g>A	56911889	Recurrent	NCBI rs 11084110			shared
a>G	56911983	Recurrent	Hematopoietic origin			
		Intron 4				
1	2	3	4	5	6	7
del T	56909591	Recurrent	Tumor specific			
c>T	56909763	Recurrent	NCBI rs 8104157			shared
g>A	56910711	Recurrent	NCBI rs 7254072			shared
g>C	56910493	Recurrent	NCBI rs 4802850			set #3
c>A	56910155	Recurrent	NCBI rs 4802849			set #3
c>G	56910154	Recurrent	NCBI rs 4802848			set #3
g>C	56910770	Recurrent	NCBI rs 1667974			set #1
t>G	56910738	Recurrent	NCBI rs 1667949			shared
a>C	56910198	Recurrent	Hematopoietic origin			
t>C	56909423	Recurrent	Hematopoietic origin			
a>G	56910219	Recurrent	Geermline origin			
inst (Ts)	56909764	Recurrent	Geermline origin			
inst (Ts)	56909761	Recurrent	Geermline origin			shared
inst (T)s	56909589	Recurrent	Geermline origin			shared
del x TTTA	56909447	Recurrent	Geermline origin			shared
ins x TTTA	56909436	Recurrent	Geermline origin			shared
c>T	56909315	Recurrent	Novel SNP			set #2
c>T	56909217	Recurrent	Geermline origin			set #2

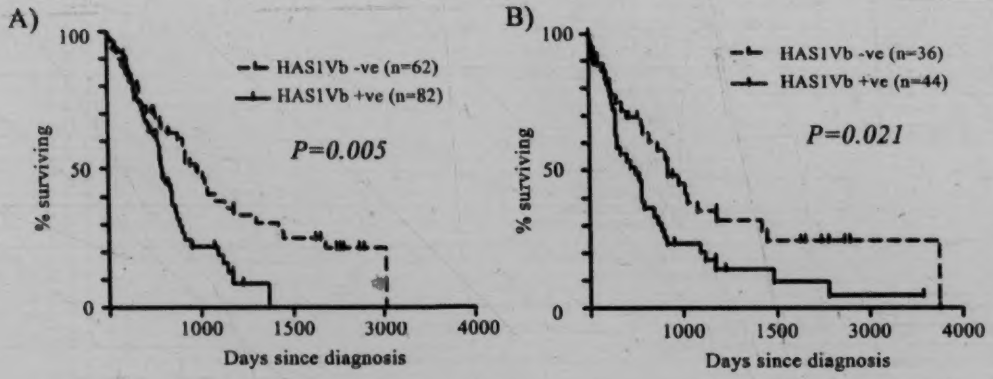
1- Nucleotide changes.
2- Changed nucleotide position in the chromosome 19q13.4.
3- Occurrence frequency of genetic variations among the patients analyzed.
4- Classification of genetic variations based on their occurrence in cell types.
5- Effects on the protein.
6- Translationally silent or missense genetic variations
7- Classifications MM clusters of GVs

Table 4-3. Overlapping GVs detected in MM and WM patients

		Exon 3			
1	2	3	4	5	6
c>T	56912080	Unique	Germline origin	Ala>Val	Missense
g>A	56912041	Unique	Germline origin	Gly>Gly	Silent
t>C	56912079	Recurrent	Hematopoietic origin	Ala>Ala	Silent
g>A	56912077	Unique	Hematopoietic origin	Cys>Tyr	Missense
t>C	56912056	Recurrent	Hematopoietic origin	Val>Ala	Missense
t>C	56912051	Recurrent	Novel SNP	Cys>Arg	Missense
t>C	56912058	Unique	Tumor specific	Cys>Cys	Silent
t>A	56912051	Recurrent	Tumor specific	Cys>Ser	Missense
		Intron 3			
t>A	56911750	Recurrent	NCBI rs 11669079		
g>A	56911831	Recurrent	NCBI rs 11084109		
g>A	56911889	Recurrent	NCBI rs 11084110		
		Exon 4			
1	2	3	4	5	6
a>T	56911346	Unique	Hematopoietic origin	Met>Leu	Missense
g>C	56911348	Unique	Hematopoietic origin	Arg>Pro	Missense
		Intron 4			
inst (T)s	56909589	Recurrent	Germline origin		
del x TTTA	56909447	Recurrent	Germline origin		
ins x TTTA	56909436	Recurrent	Germline origin		
c>T	56909217	Recurrent	Germline origin		
g>T	56910041	Unique	Hematopoietic origin		
t>G	56910738	Recurrent	NCBI rs 11667949		
g>C	56910770	Recurrent	NCBI rs 11667974		
c>G	56910154	Recurrent	NCBI rs 4802848		
c>A	56910155	Recurrent	NCBI rs 4802849		
g>C	56910493	Recurrent	NCBI rs 4802850		
g>A	56910711	Recurrent	NCBI rs 7254072		
c>T	56909763	Recurrent	NCBI rs 8104157		
Del C	56909573	Unique	Tumor specific		
t>C	56909521	Unique	Tumor specific		

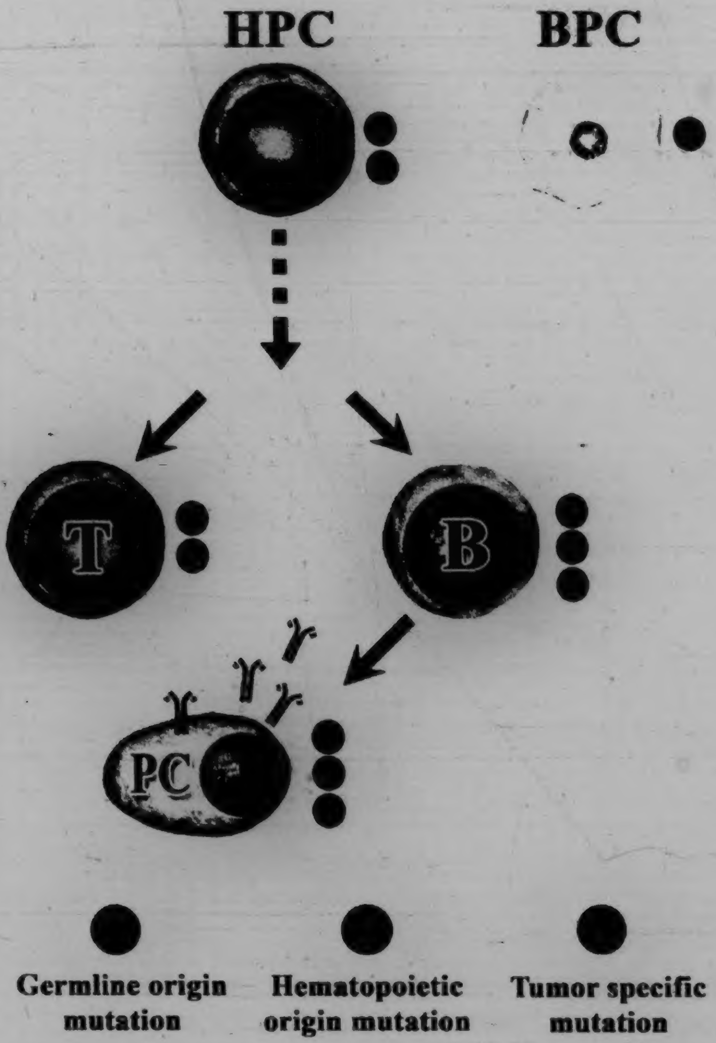
1- Nucleotide changes.
2- Changed nucleotide position in the chromosome 19q13.4.
3- Occurrence frequency of genetic variations among the patients analyzed.
4- Classification of genetic variations based on their occurrence in cell types.
5- Effects on the protein.
6- Translationally silent or missense genetic variations

Figure 4-1. Expression of HAS1Vb correlates with poor outcome of MM patients



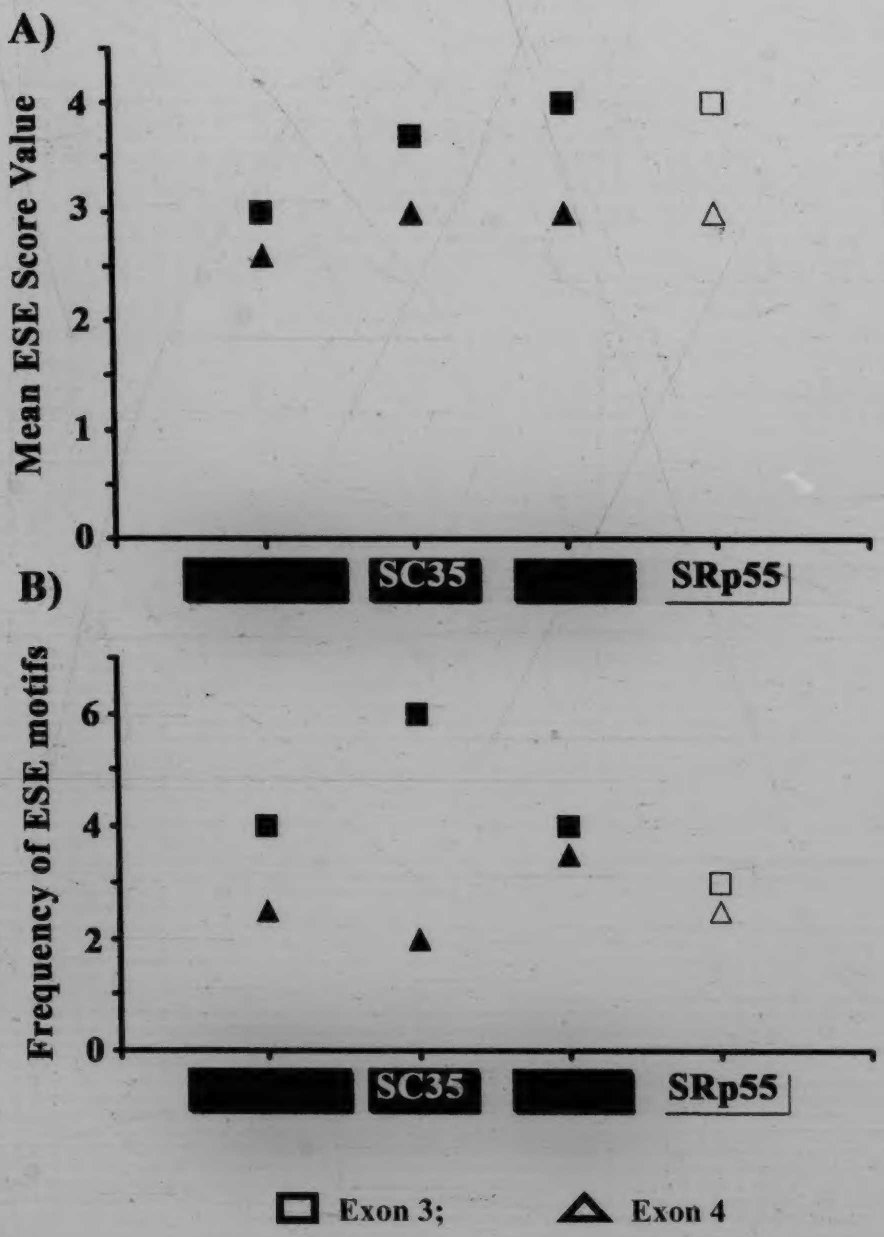
Kaplan-Meier survival distributions of MM patients with (solid curve) or without (dashed curve) detectable HAS1Vb in the PB using the Longrank Test: A) For correlation analysis samples were taken from 140 MM patients at time of diagnosis, P=0.005, HR=1.64, 95%CI=0.98-2.3, Chi-square=7.57, df=1; B) This cohort includes 80 MM patients with lytic bone lesions, P=0.021, HR=1.69, 95%CI=1.0-2.29, Chi-square=5.31, df=1

Figure 4-2. Classification of mutations detected in MM patients



Mutations identified in various types of cells from MM patients were classified as tumor specific, hematopoietic and germline origin based on their occurrence in these cells. HPC—CD34^{+}45^{low}HPCs from mobilized blood of MM patients; BEC—Buccal epithelial cell.

Figure 4-3. ***in silico*** **analysis of the impact of HAS1 mutations on binding affinity of splicing factors: High score ESE motifs in the MT HAS1 exons 3 and 4**



The four human SR proteins, SF2/ASF, SC35, SRp40 and SRp55, are color coded and shown in X-axis. The binding motif scores for these proteins are shown in Y-axis. The threshold for this proteins are SF2/ASF=1.9, SC35=2.3, SRp40=2.6 and SRp55=2.6. A) Mean motif scores of the SR proteins SF2/ASF, SC35, SRp40 and SRp55 were calculated for HAS1 exons 3 and 4. The motif scores over thresholds for exon 3 are SF2/ASF=2.9, SC35=4, SRp40=4, and SRp55=4. Motif scores over thresholds for exon 4: SF2/ASF=2.6, SC35=3, SRp40=3, and SRp55=3.

B) The frequency of SF2/ASF, SC35, SRp40 and SRp55 motifs for exon 3 and exon 4 were calculated and expressed as mean score number of motifs in 100bp exonic sequences.

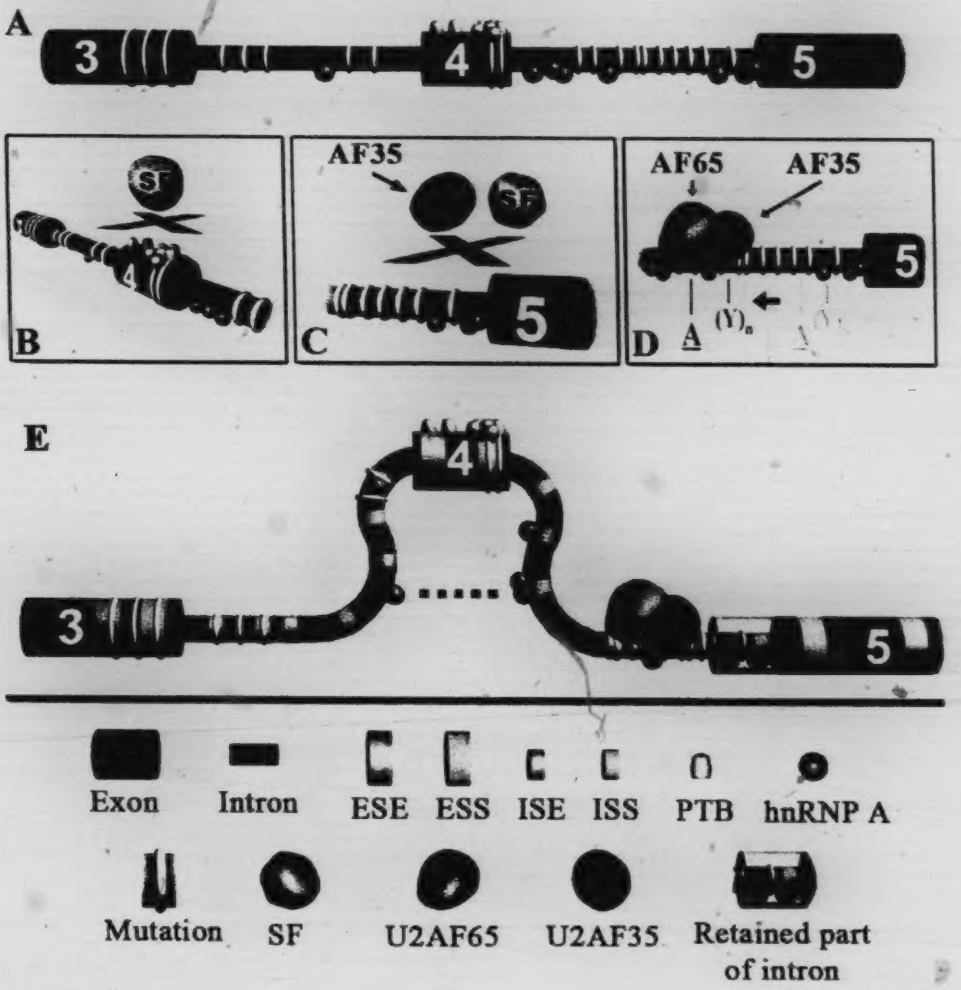
The score for each SR protein is defined based on a scoring matrix generated for each SR protein. The scoring matrices are generated based on SELEX (systematic evolution of ligands by exponential enrichment) experiments and alignment analysis. Next, the scoring matrices are calculated by the following formula described by Xiang H. et al.[53]:

$$s_i\,(a)=\log_2(f_i\,(a)+p(a)/p(a)(1+\varepsilon))$$

f_i —matrix frequency; i —position of nucleotides; ε (the Bayesian parameter)=0.5; a=nucleotides (A, C, G, U).

Details of this formula and calculations are documented in Xiang H. et al.[53]

Figure 4-4. Model: Clusters of recurrent GVs facilitate aberrant splicing of HAS1 gene in MM patients to create the intronic HAS1Vb splice variants



Using ESE finder and ASD workbench bioinformatics tools we evaluated distribution of splicing elements in HAS1 exons 3, 4 and introns 3, 4 of WT and MT sequences. Figure A demonstrates the relative distribution of recurrent mutations detected in MM patients and shows accumulation of two important splicing co-factors, hnRNP I and hnTNP A, in exon 4 and introns 3 and 4. Figure B, C, and D represent part of the HAS1 gene where the aberrations are occurring. Figure E represents the model which describes the effects of recurrent mutations on HAS1 aberrant splicing. On Figure D red letter "A" and "Y" represent activated splicing branch point (BP) and polypyrimidine tract (PPT) of splicing respectively, while gray letters: "A" and "Y" represent native BP and PPT.

Descriptions of the Model

No differences were found between WT and MT exon 3 with respect to the accumulation of hnRNPs which bind mainly splicing suppressors and promote exon exclusion. However, in MT exon 4, as compared to WT exon 4 and even MT exon 3, we predicted a massive accumulation of hnRNPs, including hnRNP I (PTB- polypyrimidine tract Binding protein), which is distributed across the entire mutated exon 4 (Figure A, B). As suggested, the binding of PTBs at several sites of an exon could cause a loop out of this exon, and subsequently these types of exons become inaccessible for the assembly of the spliceosome (Figure B, C, E)[44-47].

We did not detect any significant differences between WT and MT intron 3 with respect to Serine/Arginine rich proteins (SRs) or hnRNPs binding motif distribution. Also, no significant difference was found when BP and PPT were mapped on WT and MT intron

3. However, for MT intron 4, the existence of alternative BPs with high score were predicted. These alternative BPs are located upstream of the alternative PPT (Figure D). Additionally, splicing element analysis of WT and MT intron 4 demonstrated an accumulation of a significant number of SR and hnRNP binding motifs in MT intron 4. Among them, the most significant difference that contributes to intronic splicing of HAS1 is recruitment of U2AF65 protein by the alternative PPTs (Figure D). These predicted PPT sequences overlap with the 1st and 2nd "T" stretches and TTTA repeats of MT intron 4 were the MM clusters of GVs are located. The protein U2SF65 is known to be responsible for the recruitment of SFs to splicing BP[48-52]. Subsequently, this protein acts as a "bridge" between BP and PPT and stabilizes the spliceosomal complex necessary for the first stage of the splicing reaction.

In addition, our analysis of WT and MT intron 4 predicted a loss of a significant number of binding motifs for hnRNP proteins from MT intron 4 as compared to WT. However, MT intron 4 maintained ability to recruit hnRNP A protein which most likely contributes to the exclusion of exon 4 through its ability to dimerise with other molecules of hnRNP A located within and adjacent introns (Figure E).

Supplement Table 4-1. GVs detected in MM patients

		Exon 3				
1	2	3	4	5	6	7
Transition	c>T	56912230	Unique	Tumor specific	Pro>Leu	Missense
Transition	a>G	56912197	Unique	Tumor specific	Asp>Gly	Missense
Transition	c>T	56912186	Unique	Tumor specific	Arg>Trp	Missense
Transition	t>C	56912175	Unique	Tumor specific	Ala>Ala	Silent
Transition	c>T	56912139	Unique	Tumor specific	Asp>Asp	Silent
Transition	c>T	56912127	Unique	Tumor specific	Ser>Ser	Silent
Transition	t>C	56912089	Unique	Hematopoietic origin	Val>Ala	Missense
Transition	c>T	56912080	Unique	Germline origin	Ala>Val	Missense
Transition	t>C	56912079	Recurrent	Hematopoietic origin	Ala>Ala	Silent
Transition	g>A	56912077	Unique	Hematopoietic origin	Cys>Tyr	Missense
Transition	t>C	56912058	Unique	Tumor specific	Cys>Cys	Silent
Transition	t>C	56912056	Recurrent	Hematopoietic origin	Val>Ala	Missense
Transition	t>C	56912051	Recurrent	Novel SNP	Cys>Arg	Missense
Transversion	t>A	56912051	Recurrent	Tumor specific	Cys>Ser	Missense
Transition	g>A	56912041	Unique	Germline origin	Gly>Gly	Silent
		Intron 3				
1	2	3	4	5	6	7
Transversion	t>A	56911750	Recurrent	NCBI rs 11669079		
Transition	t>C	56911763	Unique	Tumor specific		
Transition	a>G	56911769	Unique	Germline origin		
Transition	g>A	56911813	Unique	Hematopoietic origin		
Transition	g>A	56911831	Recurrent	NCBI rs 11084109		
Transition	g>A	56911871	Unique	Hematopoietic origin		
Transition	g>A	56911889	Recurrent	NCBI rs 11084110		
Transition	g>A	56911925	Unique	Hematopoietic origin		
Transition	a>G	56911942	Unique	Tumor specific		
Transition	a>G	56911983	Recurrent	Hematopoietic origin		
Transition	g>a	56911996	Unique	Hematopoietic origin		
Transversion	g>T	56912017	Unique	Tumor specific		
Transition	c>T	56912027	Unique	Germline origin		
Transversion	a>T	56912030	Unique	Tumor specific		
Transition	t>C	56911739	unique	Tumor specific		
		Exon 4				
1	2	3	4	5	6	7
Transversion	a>T	56911346	Unique	Hematopoietic origin	Met>Leu	Missense
Transversion	g>C	56911348	Unique	Hematopoietic origin	Arg>Pro	Missense
Transition	c>T	56911343	unique	Tumor specific	Leu>Phe	Silent

		Intron 4				
1	2	3	4	5	6	7
Transition	a>G	56909186	Unique	Tumor specific		
Transition	t>C	56909196	Unique	Tumor specific		
Transition	a>G	56909216	Unique	Tumor specific		
Transition	c>T	56909217	Recurrent	Germline origin		
Transition	g>A	56909243	Unique	Germline origin		
Transition	a>G	56909280	Unique	Tumor specific		
Transition	t>C	56909285	Unique	Tumor specific		
Deletion	del C	56909308	Unique	Tumor specific		
Transition	a>G	56909349	Unique	Tumor specific		
Transition	t>C	56909389	Unique	Hematopoietic origin		
Transversion	g>T	56909402	Unique	Hematopoietic origin		
Transition	c>T	56909315	Recurrent	Novel SNP		
Transition	t>C	56909319	Unique	Tumor specific		
Transition	t>C	56909423	Recurrent	Hematopoietic origin		
Transition	g>A	56909424	Unique	Tumor specific		
Transition	g>A	56909427	Unique	Tumor specific		
Insertion	ins x TTTA	56909436	Recurrent	Germline origin		
Deletion	del x TTTA	56909447	Recurrent	Germline origin		
Transition	g>A	56909466	Unique	Tumor specific		
Transition	t>C	56909521	Unique	Tumor specific		
Transversion	t>G	56909558	Unique	Tumor specific		
Deletion	del C	56909573	Unique	Tumor specific		
Insertion	inst (T)s	56909589	Recurrent	Germline origin		
Deletion	del T	56909591	Recurrent	Tumor specific		
Transition	a>G	56909624	Unique	Tumor specific		
Transition	a>G	56909637	Unique	Tumor specific		
Transition	t>C	56909652	Unique	Hematopoietic origin		
Transition	t>c	56909738	Unique	Hematopoietic origin		
Insertion	inst (Ts)	56909762	Recurrent	Germline origin		
Transition	c>T	56909763	Recurrent	NCBI rs 8104157		
Insertion	inst (Ts)	56909764	Recurrent	Germline origin		
Transition	t>C	56909866	Unique	Tumor specific		
Transversion	g>T	56910041	Unique	Hematopoietic origin		
Transition	a>G	56910116	Unique	Hematopoietic origin		
Transversion	c>G	56910154	Recurrent	NCBI rs 4802848		
Transversion	c>A	56910155	Recurrent	NCBI rs 4802849		
Transversion	a>C	56910198	Recurrent	Hematopoietic origin		
Transition	a>G	56910219	Recurrent	Germline origin		
Transition	a>G	56910293	Unique	Germline origin		
Transition	t>C	56910366	Unique	Hematopoietic origin		
Transition	t>C	56910369	Unique	Tumor specific		
Transversion	g>T	56910395	Unique	Tumor specific		
Transition	a>G	56910410	Unique	Hematopoietic origin		
Transition	a>G	56910437	Unique	Tumor specific		
Transition	a>G	56910449	Unique	Hematopoietic origin		

1	2	3	4	5	6	7
Transition	t>C	56910451	Unique	Hematopoietic origin		
Transversion	g>C	56910493	Recurrent	NCBI rs 4802850		
Transition	a>G	56910565	Unique	Tumor specific		
Transition	a>G	56910626	Unique	Tumor specific		
Transition	g>A	56910632	Unique	Germline origin		
Transition	g>A	56910711	Recurrent	NCBI rs 7254072		
Transversion	t>G	56910738	Recurrent	NCBI rs 11667949		
Transition	g>A	56910742	Unique	Hematopoietic origin		
Transition	t>C	56910748	Unique	Tumor specific		
Transition	t>C	56910762	Unique	Tumor specific		
Transition	t>C	56910778	Unique	Tumor specific		
Transversion	a>C	56910807	Unique	Tumor specific		
Transition	c>T	56910810	Unique	Tumor specific		
Transition	a>G	56910836	Unique	Tumor specific		
Transition	t>C	56910849	Unique	Germline origin		
Transition	g>A	56910900	Unique	Hematopoietic origin		
Transition	c>T	56910905	Unique	Tumor specific		
Transversion	g>C	56910770	Recurrent	NCBI rs11667974		
Transition	a>G	56911077	unique	Tumor specific		
Transition	t>C	56911081	Unique	Germline origin		
Transition	g>A	56911083	unique	Tumor specific		
Transition	a>G	56911115	Unique	Tumor specific		
Transition	g>A	56911178	Unique	Hematopoietic origin		
Transition	a>G	56911199	Unique	Germline origin		
Transition	t>C	56911201	Unique	Hematopoietic origin		
Transition	t>C	56911210	Unique	Germline origin		
Deletion	del A	56911238	Unique	Tumor specific		
Transition	a>G	56911279	Unique	Tumor specific		
Transition	g>A	56911294	Unique	Hematopoietic origin		

1-Types of genetic variations.
2- Nucleotide changes.
3- Changed nucleotide position in the chromosome 19q13.4.
4- Occurrence frequency of genetic variations among the patients analyzed.
5- Classification of genetic variations based on their occurrence in cell types.
6- Effects on the protein.
7- Translationally silent or missense genetic variations

Chapter 4. 5. References

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Chapter 5: Conclusions

This thesis demonstrates the significant role of aberrant HAS1 gene splicing in the biology of MM and WM. Clinically, MM is considered to be a disease of the BM. MM malignant PC, which are anchored in the BM, are characterized by unique IgH VDJ rearrangements[1-6]. Molecular studies conducted by many groups, including ours, detected B cells, the precursors of BM PC, in the blood of patients with MM[1,2,5-10]. These B cells appear to be genetically similar to malignant PCs detected in the BM of patients, i.e. clonotypic IgH VDJ rearrangement found in circulating PB CD19$^+$B were identical to the VDJ rearrangement found in the BM malignant PC. This finding suggested that precursors (B cells) of malignant PCs circulate in the blood of MM patients and have the ability to home into the BM, interact with the BM microenvironment and give rise anchored malignant PCs. Thus, it was obvious to ask how these precursor B cells migrate to the BM and which molecules contribute to this process. Studies done in the Pilarski laboratory in 1996 showed that the motility of clonotypic MM B cells is mediated through interactions with HA, an important ECM molecule[11]. These malignant B cells are drug-resistant and characterized by the expression of receptors required for adhesion and motility such as CD44 and RHAMM[11-17]. CD44 and RHAMM are receptors for HA.

C. 5. 1. Hyaluronan and Hyaluronan synthases in MM and WM; differential expression and aberrant splicing of the HAS1 gene

Despite the large volume of literature on HA, experimental work to characterize the role of HA in the biology of MM or in any other disease is somewhat limited because HA is a polysaccharide that cannot be manipulated by molecular biology methods. This dilemma was resolved with the discovery of HASs, the family of enzymes that synthesize HA molecules. HASs are unique proteins that do not obey the classical dogma of one enzyme- one transferase activity. HASs have two transferase activities and perform at least six different functions to produce, assemble and translocate HA molecules into the extracellular environment or deposit an intracellular HA in the cytosol of cells, as reported by others [18-22] and documented in Chapter 2.

To understand the mechanisms of MM spread, as a first step the expression of HAS genes in MM patients was investigated. After the HAS gene profiling analysis reported in Chapter 2, it was obvious that this gene could contribute to the biology of MM. HAS3 transcripts are expressed in all cell types tested to date, including healthy donor B cells, and T cells as well as malignant B cells from a variety of B cell malignancies. The work reported in Chapter 2 shows that in contrast to HAS3, HAS1 and HAS2 are differentially expressed in MM cells, with HAS1 expression exclusive to blood CD19$^+$B cells and HAS2 expression restricted to CD38^{hi}CD45^{lo}PCs obtained from the BM of patients.

More importantly, evaluating the structure of the HAS1 gene and screening more MM patients revealed novel aberrant splice variants of HAS1, HAS1Va, HAS1Vb and HAS1Vc in MM CD19$^+$B cells (Chapters 2 and 3). Next, cloning and sequencing of the PCR products from the patients expressing HAS1 novel variants showed that HAS1Va results from alternative splicing, which deletes exon 4 from HAS1FL transcripts. Deletion of exon 4 from HAS1FL transcripts introduces an in frame shift and premature stop codon resulting in truncated HAS1Va transcript (Chapter 2). However, HAS1Vb and HAS1Vc, are results of aberrant intronic splicing which is caused by the activation of cryptic 5' or 3' splice sites in the HAS1 gene followed by the partial retention of intron 4. These aberrations cause insertion of premature stop codon in exon 5 for HAS1Vb, or at the end of exon 4 for HAS1Vc (Chapter 2). Of note, unlike HAS1Vb, HAS1Vc retains exon 4. All MM patients tested to date ubiquitously express HAS1 splice variants in various combinations (Chapter 2). Thus, the work presented in Chapter 2 of this thesis supports the view that the expression of HAS1 novel variants is characteristic of MM $CD19^{+}B$ cells, but not of BM $CD38^{hi}CD45^{low}$PCs or presumptively non-malignant T cells obtained from MM patients, nor of any cell types from healthy donors.

Statistical analysis conducted in 144 MM patients demonstrated that expression of the HAS1Vb intronic splice variant correlates with poor survival of the patients *(P=0.005)* (Chapter 4). Since expression of HAS1 and its aberrant novel variants is persistently detected in MM B cells and not BM PC and other cell populations residing in the PB or BM of patients with MM (Chapter 2), we believe that HAS1 and its novel variants can be used as a prognostic marker for risk assessment and as a biomarker to identify MM B cells in the PB of patients.

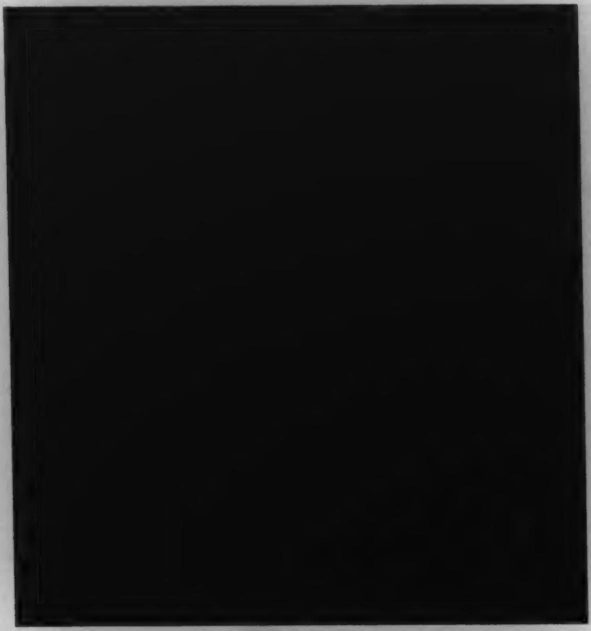
Since transitions from MGUS to MM have been detected at a rate of 1% per year, and since patients with an initial diagnosis of IgM MGUS may transform to WM, we screened MGUS and WM patients for expression profiling of HAS1 transcripts[23,24]. The expression patterns of HAS1 variants in MGUS and WM patients were similar to the expression patterns of HAS1 variants detected in MM B cells (Chapters 2, 3 and 4). Screening of WM patients demonstrated that a majority of these patients express HAS1Vb and HAS1Vc (Chapter 3). Furthermore, single cell analysis demonstrated expression of HAS1Vb transcripts in a majority of individual malignant cells (97-76%) of patients with WM, with only a minor subpopulation (3%) expressing full length HAS1[25]. Our HAS1 and variant profiling analysis demonstrated that expression of HAS1 splice variants was detected in MM, MGUS and WM patient samples taken at the time of diagnosis or relapse (Chapters 2 and 3). These findings suggest that the HAS1 gene is involved in early stages of myelomagenesis as well as contributing to progression of the disease.

Studies conducted on other genes have shown that the retention of introns during splicing, the type of aberration, that generates HAS1Vb and HAS1Vc transcripts in MM and WM patients, is uncommon, occurring in only 6% of alternatively spliced genes[26,27]. The same type of splicing aberration has been detected in CDKN2A, MDM2, ATM and CD44 genes (ASD-The Alternative Splicing Database; AltSplice-Human release 2, April 2005). It appears that in most cases, partial retention of introns is characteristic of the genes associated with a malignant phenotype. By analogy, aberrant intronic splicing of HAS1 may reflect fundamental aspects of disease biology in MM and WM. Thus, our own work, coupled with these observations from the literature, suggest that HAS1 family members, particularly HAS1Vb, are clinically and biologically significant in MM and WM, a speculation that was confirmed by the survival analysis performed in MM patients (Chapter 2 and 4).

C. 5. 2. HAS1 splice variant transcripts encode functional proteins

As indicated in chapter 2, based on western blotting and alignment analysis, HAS1 and its variants are likely to produce their corresponding proteins. The functional capabilities of the translated proteins were verified by evaluating the ability of ex-vivo malignant B cells to synthesize extracellular and/or intracellular HA (Chapter 2). As reported in Chapters 2-4, malignant B cells from MM and WM patients express HAS1 variants and HAS1FL in combination with HAS3. In MM patients the expression of HAS1Va, with or without HAS1FL transcripts and with HAS3, is associated with the ex-vivo synthesis of extracellular HA around MM B cells (Chapter 2). In contrast, MM B cells expressing HAS1Vb in combination with HAS3 have readily detectable intracellular HA. Intracellular HA has also been detected in BM B cells from patients with WM expressing HAS1Vb transcripts in combination with HAS3 but in the absence of HAS1FL (Figure 5-1). MM B cells expressing HAS1FL and HAS3 in the absence of a splice variant transcript lacked both extracellular and intracellular HA, suggesting that the splice variants are essential for extracellular and/or intracellular HA synthesis in MM cells. The role of HAS1FL in this process, if any, appears to be dispensable, but the contributions of HAS3 remain unknown. It may be that, HAS3 facilitates HA synthesis by HAS1 splice variants, a speculation being currently addressed in the Pilarski laboratory using HAS1 transfectants. Thus, our work leads to the conclusion that the only source of intracellular HA detected in MM and WM B cells is synthesized by HAS1Vb protein encoded by HAS1Vb transcripts. These findings suggest that HAS1 splice variant transcripts encode functional proteins that are able to produce and assemble HA molecules.

Figure 5-1. Intracellular HA in WM CD20+B cells synthesized by HAS1Vb



CD20$^+$B cells were obtained from the BM of patients with WM. Cells were cultured for 48h, treated with hyaluronidase to remove extracellular HA and than incubated with biotinylated HA binding protein as described in Chapter 2. Cells were fixed with 4% paraformaldehyde for 15 min at 4^{0}C. Next HA binding to B-HABP was visualized using streptavidin Alexa 594 (Red). Blue color represents DAPI staining of the nucleus. The controls for this staining are presented in Chapter 2, Figure 2-6. The 3D projection of WM CD20$^+$B cell was obtained utilizing an Axiovert 100M LSM (Laser Scanning microscope) 510. The staining pattern shown here suggests that intracellular HA is distributed along the cell cytoskeleton and perinuclear compartment of WM CD20$^+$B cells.

In support of the functional analysis, alignment and protein motif screening analysis showed that all three variants of HAS1 retained motifs which encode the glucosyltransferase activity required to produce HA molecules. Also, the conserved amino acids which determine size of HA molecules are present in the sequences of HAS1Va, HAS1Vb and HAS1Vc (Figure 5-2). The folding ability of HAS1 variant proteins were evaluated using *in silico* methods, including the PSIPRED V2.4 server, MEMSAT V3 and mGenTHREADER. The PSIPRED V2.4 bioinformatics tool predicts the secondary structure of proteins based on the analysis obtained from PSI-BLAST (Position Specific Iterated-BLAST from NCBI). MEMSAT V3 is an all-helical membrane protein prediction method, which predicts transmembrane topology of a protein based on a set of transmembrane proteins of known topology. Accuracy of predicting the structure of transmembrane proteins and their location within a membrane using PSIPRED V2.4 and MEMSAT V3 tools was 78%. A widely used folding recognition method, mGenTHREADER, allows the target sequence (the protein sequence for which the structure is being predicted) to be threaded through the backbone structures of template proteins grouped as the fold library. In addition to its original algorithm, mGenTHREADER uses a PSI-BLAST profile and secondary structure predicted by PSIPRED as inputs to generate folding profiles of the protein under investigation. Use of this approach allowed us to conclude that all three variants of HAS1 are able to fold properly. As an example, here we compared the fold profile of HAS1FL to the folding profile of HAS1Vb (Figure 5-3). As this Figure demonstrates, even though one domain is missing in the HAS1Vb folding profile the folding pattern of this protein has not been altered. This finding is supported by the preliminary results obtained from the transfection analysis of HAS1 variant GFP constructs in HeLa cells (Figure 5-4).

Figure 5-2. HAS1 novel variants retain glycosyltransferase activity

```
                  *        20         *        40         *        60         *        80
HAS1FL : MRQQDAPKPTPAARRCSGLARRVLTIAFALLILGLMTWAYAAGVPLASDRYGLLAFGLYGAFLSAHLVAQSLFAYLEHRRVAAAA :  85
HAS1Va : MRQQDAPKPTPAARRCSGLARRVLTIAFALLILGLMTWAYAAGVPLASDRYGLLAFGLYGAFLSAHLVAQSLFAYLEHRRVAAAA :  85
HAS1Vb : MRQQDAPKPTPAARRCSGLARRVLTIAFALLILGLMTWAYAAGVPLASDRYGLLAFGLYGAFLSAHLVAQSLFAYLEHRRVAAAA :  85
HAS1Vc : MRQQDAPKPTPAARRCSGLARRVLTIAFALLILGLMTWAYAAGVPLASDRYGLLAFGLYGAFLSAHLVAQSLFAYLEHRRVAAAA :  85
         MRQQDAPKPTPAARRCSGLARRVLTIAFALLILGLMTWAYAAGVPLASDRYGLLAFGLYGAFLSAHLVAQSLFAYLEHRRVAAAA

             *       100         *       120         *       140         *       160         *
HAS1FL : RGPLDAATARSVALTISAYQEDPAYLRQCLASARALLYPRARLRVLMVVDGNRAEDLYMVDMFREVFADEDPATYVWDGNYHQPW : 170
HAS1Va : RGPLDAATARSVALTISAYQEDPAYLRQCLASARALLYPRARLRVLMVVDGNRAEDLYMVDMFREVFADEDPATYVWDGNYHQPW : 170
HAS1Vb : RGPLDAATARSVALTISAYQEDPAYLRQCLASARALLYPRARLRVLMVVDGNRAEDLYMVDMFREVFADEDPATYVWDGNYHQPW : 170
HAS1Vc : RGPLDAATARSVALTISAYQEDPAYLRQCLASARALLYPRARLRVLMVVDGNRAEDLYMVDMFREVFADEDPATYVWDGNYHQPW : 170
         RGPLDAATARSVALTISAYQEDPAYLRQCLASARALLYPRARLRVLMVVDGNRAEDLYMVDMFREVFADEDPATYVWDGNYHQPW

                180         *       200         *       220         *       240         *
HAS1FL : EPAAAGAVGAGAYREVEAEDPGRLAVEALVRTRRCVCVAQRWGGKREVMYTAFKALGDSVDYVQVCDSDTRLDPMALLELVRVLD : 255
HAS1Va : EPAAAGAVGAGAYREVEAEDPGRLAVEALVRTRRCVCVAQRWGGKREVMYTAFKALGDSVDYVQVCDSDTRLDPMALLELVRVLD : 255
HAS1Vb : EPAAAGAVGAGAYREVEAEDPGRLAVEALVRTRRCVCVAQRWGGKREVMYTAFKALGDSVDYVQVCDSDTRLDPMALLELVRVLD : 255
HAS1Vc : EPAAAGAVGAGAYREVEAEDPGRLAVEALVRTRRCVCVAQRWGGKREVMYTAFKALGDSVDYVQVCDSDTRLDPMALLELVRVLD : 255
         EPAAAGAVGAGAYREVEAEDPGRLAVEALVRTRRCVCVAQRWGGKREVMYTAFKALGDSVDYVQVCDSDTRLDPMALLELVRVLD

           260         *       280         *       300         *       320         *       340
HAS1FL : EDPRVGAVGGDVRILNPLDSWVSFLSSLRYWVAFNVERACQSYFHCVSCISGPLGLYRNNLLQQFLEAWYNQKFLGTHCTFGDDR : 340
HAS1Va : EDPRVGAVGGDVRILNPLDSWVSFLSSLRYWVAFNVERACQSYFHCVSCISGSLGTPPGPAATQRRPRPSCGG------------ : 328
HAS1Vb : EDPRVGAVGGDVRILNPLDSWVSFLSSLRYWVAFNVERACQSYFHCVSCISGPLESCPGPREHAMMPSFLAFVQVHLQVPLLLRE : 340
HAS1Vc : EDPRVGAVGGDVRILNPLDSWVSFLSSLRYWVAFNVERACQSYFHCVSCISGPLGLYRNNLLQQFLEAWYNQKFLGTHCTFGDDR : 340
         EDPRVGAVGGDVRILNPLDSWVSFLSSLRYWVAFNVERACQSYFHCVSCISGpLg        q

                  *       360         *       380         *       400         *       420
HAS1FL : HLTNRMLSMGYATKYTSRSRCYSETPSSFLETLSQQTRMSKSYFREVLYNALWWHRHHAWMTYEAVVSGLFPFFVAATVLRLFYA : 425
HAS1Va : ------------------------------------------------------------------------------------- :   -
HAS1Vb : ALVLPAVAEPADTLVQTVLP----------------------------------------------------------------- : 360
HAS1Vc : HLTNRMLSMGYATK----------------------------------------------------------------------- : 354
         l           t

             *       440         *       460         *       480         *       500         *
HAS1FL : GRPWALLWVLLCVQGVALAKAAFAAWLRGCLRMVLLSLYAPLYMCGLLPAKFLALVTMNQSGWGTSGRRKLAANYVPLLPLALWA : 510
HAS1Va : ------------------------------------------------------------------------------------- :   -
HAS1Vb : ------------------------------------------------------------------------------------- :   -
HAS1Vc : ------------------------------------------------------------------------------------- :   -

                520         *       540         *       560         *
HAS1FL : LLLLGGLVRSVAHEARADWSGPSRAAEAYHLAAGAGAYVGYWVAMLTLYWVGVRRLCRRRTGGYRVQV : 578
HAS1Va : -------------------------------------------------------------------- :   -
HAS1Vb : -------------------------------------------------------------------- :   -
HAS1Vc : -------------------------------------------------------------------- :   -
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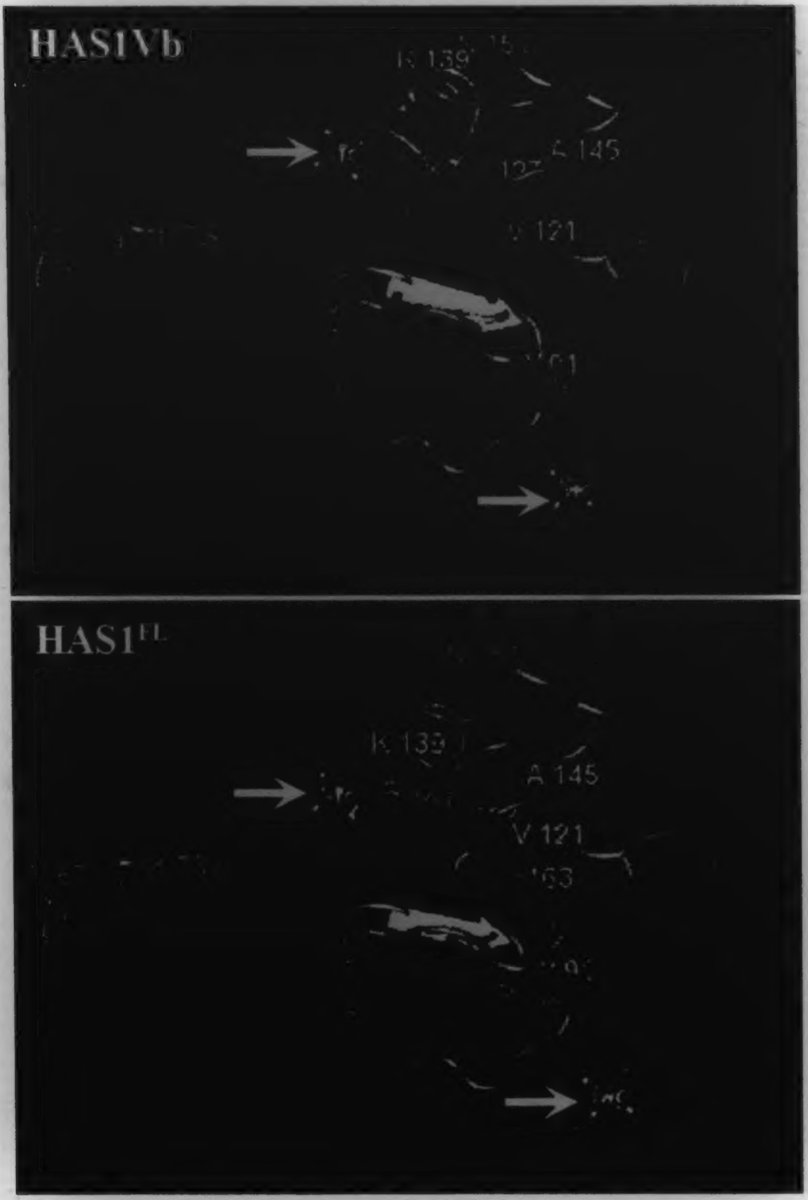
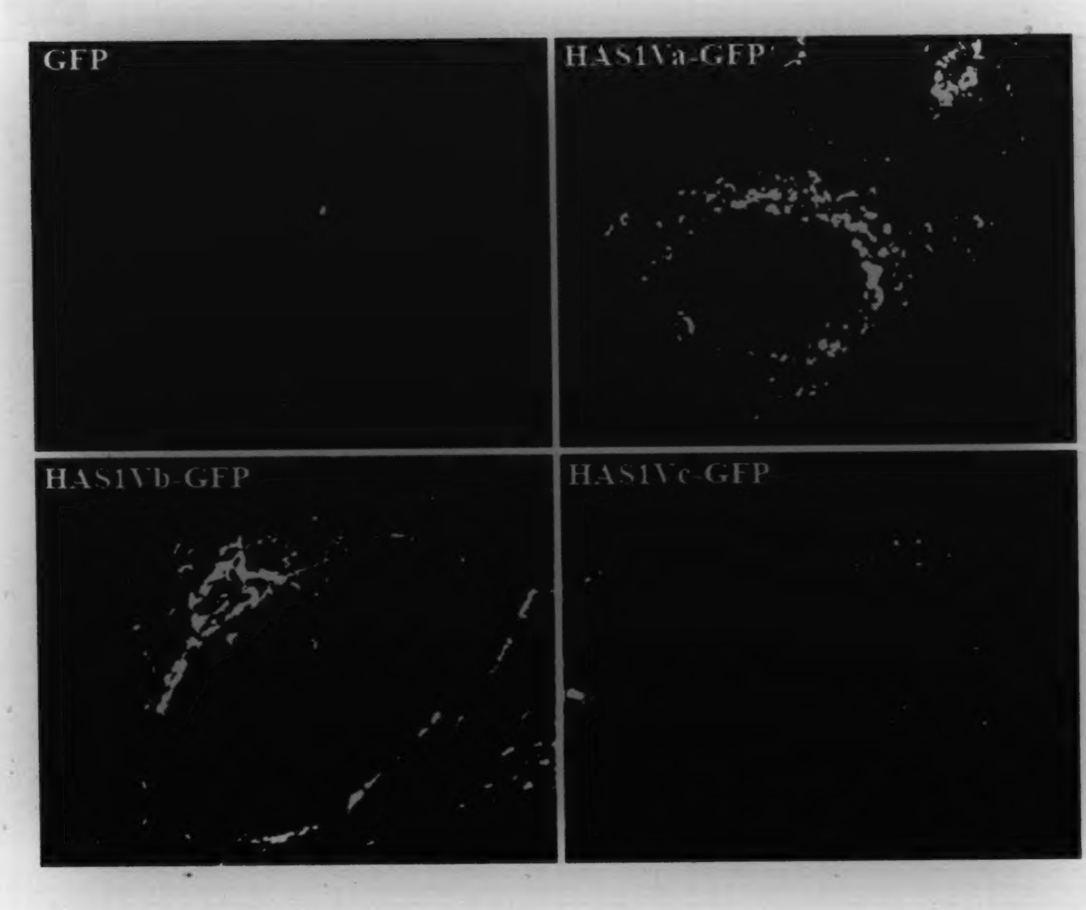
Protein sequence alignment of HAS1 family members, HAS1Va, HAS1Vb and HAS1Vc. Amino acid sequences of HAS1 novel variants are aligned with reference to HAS1^{FL}. On the figure the residues highlighted in gray represent the glycosyltransferase motif while the residues highlighted in black represent conserved stretch of the enzymatically active central loop of the HAS protein. This stretch of the HAS protein is conserved among all HASs identified to date and is known to be responsible for the synthesis of HA molecules since this central loop of the HAS protein includes the glycosyltransferase motif. This multiple sequence alignment plot shows the retention of the glycosyltransferase motif among the novel splice variants of HAS1. The sequence alignment shown in the figure was created using GeneDoc (multiple sequence alignment editor, analyzer and shading utility) software. The central loop and glycosyltransferase motif were mapped based on the motif scanning analysis of HAS1^{FL} and HAS1 novel variants using ExPASy-PROSITE (Database of protein families and domains) database.

Figure 5-3. Predicted HAS1Vb protein folding



In silico folding analysis of HAS1Vb shows retention of the enzymatically active central loop of HAS1Vb. The folding of the domains, including the glycosyltransferase domain comprising the central loop of HAS1Vb, are identical to corresponding HAS1FL domains. On this Figure, helices are shown in green, strands in yellow, coils in blue and pink dots represent solvents. Arrows indicate Mg ions. The helix with the red dot on HAS1FL image is absent from HAS1Vb image. The HAS protein folding was visualized using Molecular Graphics Visualization tool RasTop.

Figure 5-4. Localization of HAS1 variant proteins in HeLa cells



HeLa cells (a human epithelioid cervical carcinoma cell line) were transiently transfected with HAS1Va-, HAS1Vb- and HAS1Vc-GFP constructs. All novel splice variants of HAS1 produced proteins as it is shown on Figure 4. We detected very strong intracellular vesicular accumulation of HAS1Va-GFP proteins in HeLa cells. In contrast, HAS1Vb and HAS1Vc proteins were accumulated in perinuclear structures with no vesicular localization. The most interesting distribution pattern was observed when HeLa cells were transiently transfected with HAS1Vb-GFP construct, which showed a fine filamentous network of GFP staining that stretches from the nuclear membrane to the cell plasma membrane and forms a "cage" around the cell nucleus. This intriguing distribution pattern suggests that HAS1Vb may co-localize with the cell cytoskeletal elements. Figure 4 also includes an image which demonstrates distribution of GFP protein in every compartment of HeLa cells transfected with GFP alone. Transfection of HeLa cells with GFP only provided a control for the transfection. The functional interaction of these novel variants with each other and with other intracellular elements are currently being investigated in the Pilarski laboratory.

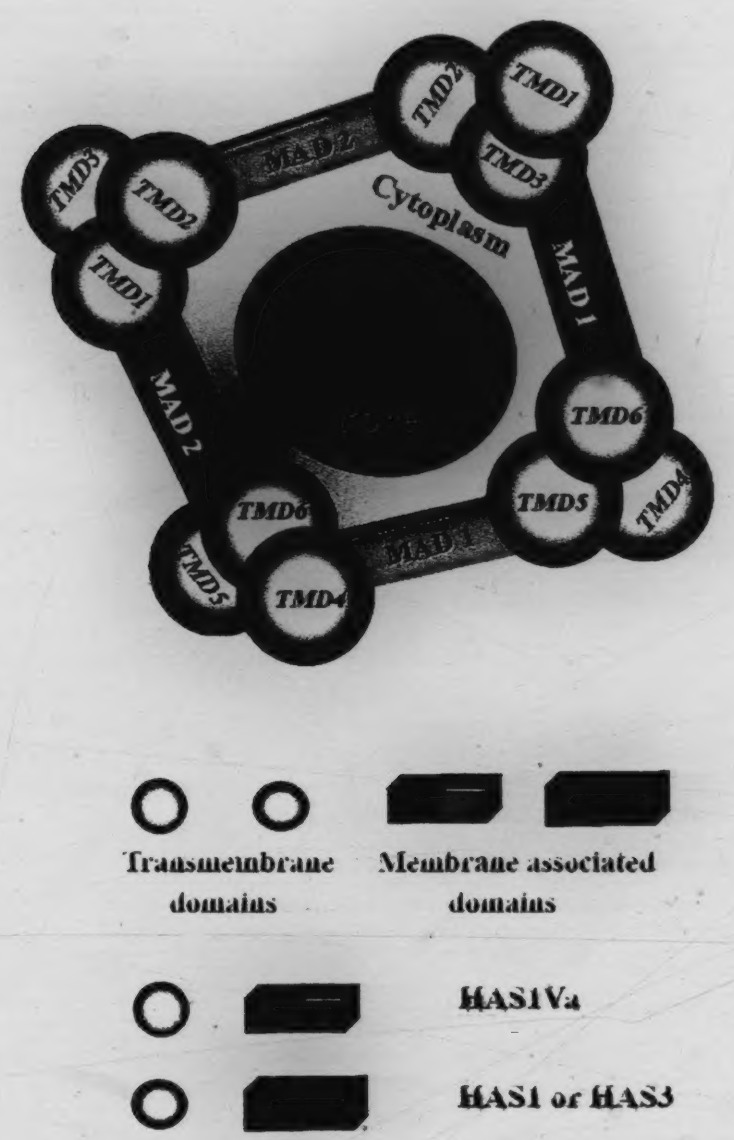
C. 5. 3. Extracellular HA and HAS1Va

Recently, it has been demonstrated that interactions between endogenous HA and malignant cells regulate multidrug-resistance of these cells[28]. Also, Baumgartner et al. showed that removal of the HA matrix from the cell plasma membrane of malignant cells, accomplished by hyaluronidase treatment, improved the effectivness of various chemotherapeutic agents on these cells[29]. Analysis of MM B cells showed that cells surrounded by extracellular HA halos exhibited long microvilli-like protrusion (Chapter 2, Figure 2-5 B), suggesting that these cells may be motile (as shown independently by Masellis Smith et al.) and that synthesis of HA molecules contributes to the motility of these cells[11]. Recently, Kultti et al. demonstrated that HAS3 proteins are localized in the protrusions of cells where the proteins which promote drug resistance also accumulate[30]. Thus, the possibility exists that the HA molecules localized in the protrusions detected around MM CD19$^+$B cells expressing HAS1Va in combination with HAS3 contribute to the motility of these cells. Furthermore, these observations may help to explain why MM B cells are drug-resistant. Also, depletion of extracellular HA by hyaluronidase treatment dramatically reduced mechanical strength of the plasma membrane[30]. Thus, extracellular HA detected around circulating MM CD19$^+$B cells may act as an extracellular cytoskeleton for these cells, thereby providing support for the membrane of MM B cells to maintain their malignant morphology.

C. 5. 4. Translocation of extracellular HA into the ECM

HASs are highly hydrophilic proteins that paradoxically translocate HA molecules into the ECM through the hydrophobic plasma membrane. In 1999 Tlapak-Simmons et al., in their study investigating *Streptococcal HAS,* suggested the formation of pore-like structures in the membrane of bacteria through interactions of the *Streptococcal HAS* molecules with the membrane cardiolipins[31]. Similar to this finding, since HAS1 novel variants are expressed exclusively in malignant B cells, in combination with each other and/or HAS1 and HAS3, the possibility exist that HAS1 novel variants form homo- or hetero-dimers with each other, with HAS1FL and/or with HAS3. The formation of homo- and/or hetrodimeric combinations would solve the HA translocation paradox since homo and/or heterodimers could accumulate transmembrane domains sufficient for the formation of pore-like structures. In this manner, HA chains synthesized by HAS1Va in combination with, for example, HAS3 and/or HAS1FL can be extruded into the ECM through these pores. This model is depicted in Figure 5-5. However, details of this model must await further experimental work to study the localization and co-localization of HAS1 novel variants and HAS3 in transfected cell lines derived from MM and/or WM patients.

Figure 5-5. Model for translocation of HA into the ECM



Schematic visualization of homo- and/or hetero-dimers of HAS1Va and/or HAS1FL and HAS3 formed into the membrane. The pore generated by homo- or hetero-dimers is visible from the outside of the cell. The light blue color represents cytoplasm, while the dark blue color represents the pore itself. The membrane-associated domains of the HAS1Va, HAS1 and/or HAS3 are labeled as MAD and trans-membrane domains are labeled as TMD. This model has been generated based on the predicted topological structures or HAS1Va, HAS1 and/or HAS3.

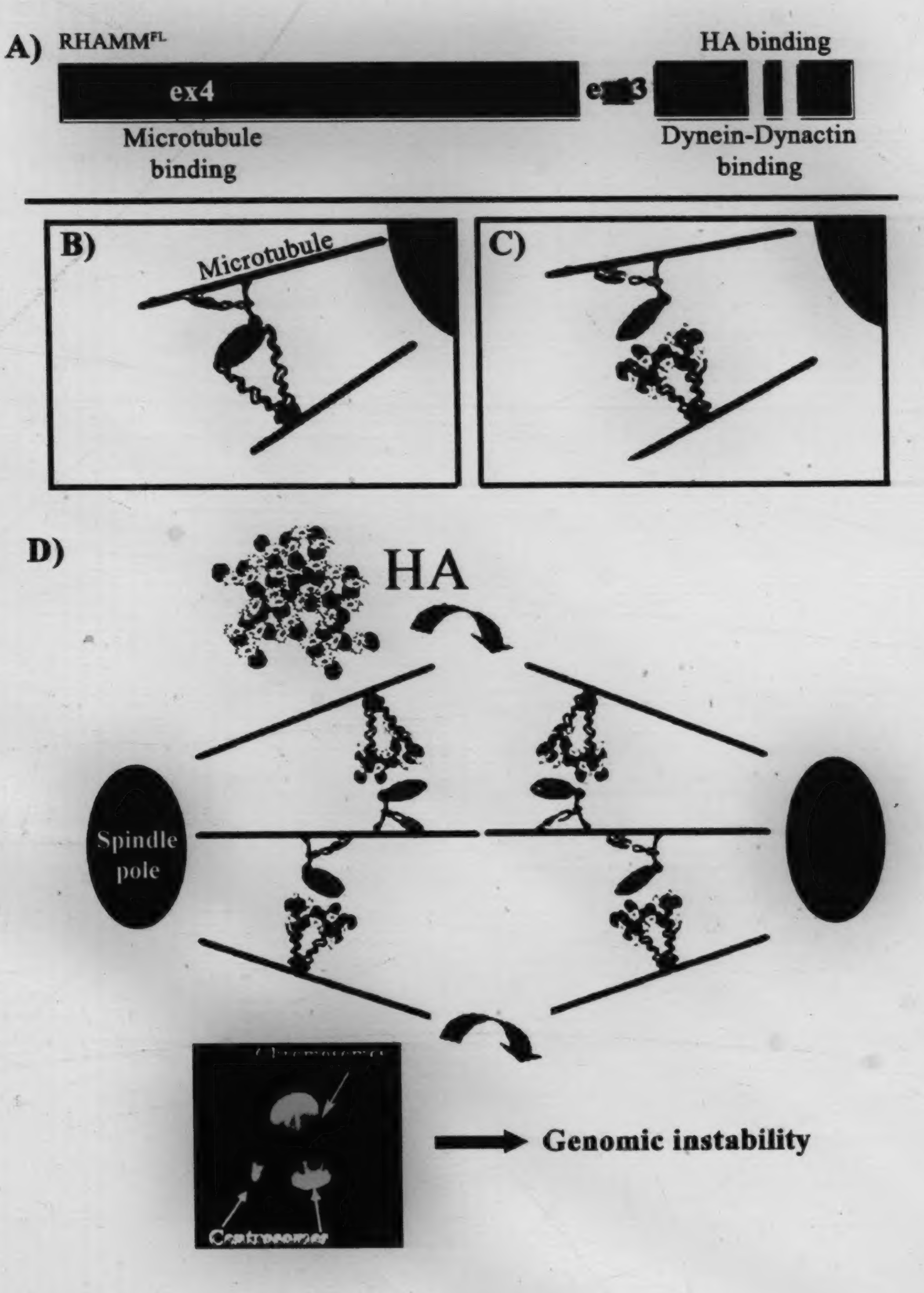
C. 5. 5. Intracellular HA

In addition to extracellular HA, intracellular HA has been detected in nucleoli, the nuclear periphery, rough endoplasmic reticulum and caveolae[19,20,32-37]. An extensive review published by Hascall et al. describes the role of intracellular HA in mitosis and inflammation[38]. Furthermore, intracellular HA has been detected throughout cell division[38]. Evanko et al., in their recent study, documented interaction of intracellular HA with intracellular RHAMM[19] However, the exact function of intracellular HA remains unclear and studies referenced above are not conclusive. Also, the source of these molecules needs to be determined.

The HA binding receptor RHAMM, in addition to its role as a cell surface receptor for HA, is also a centrosomal protein that maintains stability of the mitotic spindle. Depletion of intracellular RHAMM leads to the formation of tri- or tetra-polar mitotic spindles leading to dysregulation of mitosis[39]. Overexpression of exogenous RHAMM also induces aberrant spindle architecture. Thus, increased RHAMM expression may induce extensive genetic instability in cancers, particularly in MM, as proposed elsewhere[39-41].

Interestingly, the HA binding domain of RHAMM at the C-terminus overlaps with the dynein-dynactin binding domain of this molecule. Maxwell et al. demonstrated that disruption of the interaction between RHAMM and dynein-dynactin leads to a multiple spindle formation which in turn promotes missegregation of chromosomes promoting aberrant mitosis[39]. This phenomenon was demonstrated though blocking of intracellular RHAMM by intracellular injection of anti-RHAMM antibody. It is intriguing to speculate that the intracellular HA produced by HAS1Vb interacts with intracellular RHAMM most likely at the C-terminus of the receptor where the HA motif overlaps with the dynein-dynactin binding domain. Interaction of intracellular HA with RHAMM may disrupt the interaction of RHAMM with microtubules and as a result promote chromosomal missegregation in MM (Figure 5-6). Thus, HAS1 and its variants, in concert with RHAMM, may contribute to genomic instability in MM. Furthermore, novel variant(s) of HAS1 may form heterodimers with intracellular RHAMM, thereby sequestering RHAMM in the membrane where it can activate the *ras* signaling pathway.

Figure 5-6. Intracellular HA may facilitate aberrant mitosis



A model for HA and HAS facilitated oncogenesis. A) The HA binding domain of RHAMM overlaps the dynein-dynactin binding domain. B) RHAMM binds to microtubules via dynein-dynactin and helps to maintain spindle pole integrity. C) Intracellular HA, produced by novel variants of HAS1 may disrupt RHAMM-dynein-dynactin interactions. D) Intracellular HA may promote aberrant mitosis.

C. 5. 6. Aberrant splicing of HAS1

Although alternative splicing events are normal phenomena that contribute to protein diversity in humans, more than a dozen human cancers are associated with abnormalities in alternative splicing. As mentioned in introductory chapter the products of alternative splicing yield proteins with different physiological functions. These proteins could act as dominant-negative inhibitors and will be able to compromise the normal function of wild type proteins. Furthermore, alternative splice variants of a given gene either could cause disease or be involved in disease development and progression. Further, they may be valuable cancer markers. Examples of aberrant splicing include the BRCA1 gene in breast and ovarian cancer, MDM2 and CD44 in various types of cancers, the CDKN2A, which predisposes individuals to melanoma, and XPG DNA repair endonuclease gene which is associated cancer-prone disorders xeroderma pigmentosum (XP) and XP-Cockayne syndrome complex [42-51]. In addition several examples reported in literature suggest that alternative splice variants are overexpressed as hyper-oncogenic proteins. These proteins often correlate with poor prognosis. Thus, alternative and/or aberrant splicing m ay play a profoundly important role in the origins and progression of cancer.

As mentioned in previous chapters, pre-mRNA processing, which occurs in the nucleus of the cell, is a complex process which includes pre-mRNA splicing. Splicing of a given gene requires activation of more than 100 proteins, including splicing factors and 5 small nuclear ribonucleoprotein particles[52]. Specificity of the splicing is defined by classical splicing elements including the 5’ and 3’ splicing sites, branch point and polypyrimidine tract of splicing. However, evidence reported in the literature suggests the importance of cis-splicing elements, such as exonic and intronic splicing enhancers/suppressors and polypyrimidine tracts of splicing in this process[53,54]. Furthermore, mutations occurring in cis-splicing elements, alone or in combination with an aberrant expression of splicing factors play a significant role in aberrant splicing[53,55,56]. However, additional genetic variations (GVs) on polypyrimidine tracts and on the splicing branch points are required to activate cryptic splice sites and achieve aberrant splicing[54,57-59]. The consequences of the mutations occurring within the sequences of splicing elements are exon skipping and intron retention[56,60].

The expression of HAS1 and its novel variants in malignant MM or WM cells (Chapters 2 and 3) and the correlation of the HAS1Vb intronic variant with poor survival of MM patients (Chapter 2) in the context of the observations by others as discussed above, sparked my interest in investigating the mechanisms underlying the partial retention of intron 4 in HAS1Vb and HAS1Vc transcripts from MM and WM patients. Understanding these processes might provide valuable insights into the biology of these diseases.

As a first step to address this question, genomic HAS1 sequences in the vicinity of retained intron 4 and excluded exon 4 of the HAS1 gene were screened against the NCBI database to identify any SNPs reported on exons 3, 4 and 5 and introns 3 and 4. This observation in combination with the bioinformatics analysis previously reported by us, identified HAS1 833A/G SNP in exon 3 as a candidate SNP for genotyping[61]. Extensive genotyping analysis conducted in MM and WM patients showed increased homozygosity for HAS1 833 allele "G" in these patient populations (*P=0.000002* for MM and *P=0.00031* for WM; Chapters 3 and 4). Since 64% of healthy donors are also homozygous for HAS1 833 allele "G", homozygosity of the HAS1 833 A/G SNP alone cannot explain the aberrant splicing patterns detected in MM and WM. These observations suggest that additional GVs are likely to be involved in the generation of aberrant HAS1 gene splicing. Nevertheless, HAS1 833G/G homozygosity is strongly correlated with the presence of intronic splice variants, particularly HAS1Vb transcripts in WM patients (Chapter 3). WM patients expressing HAS1Vb and HAS1Vc were exclusively homozygous for HAS1 833 allele "G" (Chapter 3). Our genotyping and bioinformatics analysis, through which we identified classical and cis- splicing elements, demonstrate that the HAS1 833 A/G SNP, located in exon 3, is necessary but not sufficient for the retention of intronic sequence in HAS1Vb and HAS1Vc transcripts (Chapter 3 and 4).

Next, the region of alternatively spliced exon 4 and retained intron 4 of the HAS1 gene was sequenced to identify additional potential genetic variations associated with HAS1 splicing. As reported in chapters 3 and 4, these extensive sequencing analyses identified three types of inherited and acquired HAS1 genetic variations, classified based on their occurrence in the cell populations obtained from MM and WM patients. Overall, any given patient had approximately 22 GVs in the HAS1 gene segments analyzed. These genetic variations include **tumor specific** GVs detected in PB B and BM PC cells from either MM and WM patients, **hematopoietic origin** GVs identified in PB B and T cells, BM PC, and CD34$^+$CD45^{low}HPCs (hematopoietic progenitor cells) from G-CSF mobilized blood of MM patients, and **germline origin** GVs detected in all analyzed cell populations of the patients including buccal epithelial cells. Some of these genetic variations were recurrent among the patients, while others were specific to individual patients.

Segments of the HAS1 gene from malignant B cells and PCs, and non-malignant T cells were sequenced first, to determine whether genetic variations detected in tumor cells obtained from patients were disease related. These experiments identified genetic variations specific to tumor cells i.e. detected in B and PCs, as well as genetic variations that were shared among malignant B cells, PCs and non-malignant T cells. To identify the origin of the shared genetic variations detected in malignant B cells, PCs and non-malignant T cells, segments of the HAS1 gene from buccal epithelial cells of the patients were sequenced. Unexpectedly, some GVs detected in B and T cells were absent from buccal epithelial cells of the same patients. Since buccal epithelial cells are almost certainly unrelated to the malignant clone, this observation indicated that some GVs were characteristic of hematopoitic cells or tumor cells, but absent from the germline of the patient. Perhaps the GVs in the hematopoietic lineage cells (genetic variations detected in B cells, PCs and T cells but not in buccal epithelial cells) are "premalignant" somatic GVs, and/or results of mutational events, that accumulate at the earliest stages of oncogenesis, eventually culminating in malignant B cell clones in context of tumor specific GVs. Therefore, we sequenced HAS1 gene segments from hematopoietic progenitor cells derived from G-CSF mobilized blood of MM patients. This was not feasible for WM because in Alberta, WM patients are not offered high dose chemotherapy with stem cell rescue.

Sequencing of HAS1 gene segments from hematopoietic progenitor cells of patients with MM demonstrated that genetic variations detected in B cells, PCs and T cells were also present in hematopoietic progenitor cells, but were absent from buccal cells. These genetic variations were classified as hematopoietic origin GVs. Their absence from buccal cells indicates that they are not likely to be part of the patient germline and thus, they are not inherited predispositions. These GVs appear to be acquired during hematopoietic cell differentiation at some point in the lifetime of each individual, and are thus viewed as somatic or acquired GVs. It is intriguing that some of the HAS1 GVs detected in hematopoietic progenitor cells from blood of MM patients were also present in T and B cells from WM patients, a related but different malignancy. This finding further supports the idea that these genetic variations are results of mutational selection leading to malignant disease.

Additionally, we detected germline origin GVs with increased homozygosity of mutated alleles in analyzed patients as compared to healthy donors. Furthermore, when genetic variations detected in MM and WM patients were compared to each other, we identified overlapping GVs present in both types of patient (Chapter 4). Based on this observation some of the genetic variations originally defined as unique were reclassified as recurrent.

Additionally, mutational analysis demonstrated that these GVs were distributed on the HAS1 gene in a nonrandom manner as clusters in the vicinity of splicing elements. Bioinformatics analysis predicted involvement of these mutational clusters in HAS1 aberrant splicing that gives rise to assembly of HAS1Vb and HAS1Vc transcripts (Chapter 4). Thus, GVs which contribute to aberrant splicing of the HAS1 pre-mRNA transcripts can be evaluated as splicing mutations.

Thus, this work supports the view that genetic variations associated with aberrant splicing of the HAS1 gene accumulate at an early stage of oncogenesis in MM and WM. Because hematopoietic progenitor cells of patients have activated self-renewal machinery, it seems reasonable to propose that accumulation of GVs related to aberrant splicing of the HAS1 gene first occurring in these cells and are then passed down through lymphoid differentiation, ultimately to the parent B cells that give rise to the malignant clones of MM or WM as they “survive” mutational selection events throughout the hematopoietic lineage.

As studies reported in Chapter 2 and 3 suggest, expression of HAS1Vb transcripts is characteristic of MM and WM B cells and has not been detected in other hematopoietic lineage cells such as T cells from these patients. Thus, hematopoietic origin GVs are necessary but not sufficient for HAS1 gene splicing. Based on the observations reported here, the effects of hematopoietic origin genetic variations on HAS1 splicing are manifest in MM malignant cells in the context of additional GVs, which are acquired in circulating malignant B cells during their clonal expansion and final differentiation to malignant

PCs. Finally, it is intriguing to speculate that, similar to certain types of leukemias such as acute lymphoblastic leukemia, chronic myeloid leukemia, and myelodysplastic syndromes (MDS), initiation events that contribute to MM pathogenesis may arise in the hematopoietic cell lineage and particularly in progenitor cells[62-64].

Additionally, genetic variations reported in chapter 4 include 27 overlapping GVs detected in MM and WM patients, the majority of which are of either hematopoietic or germline origin. This evidence, among other findings reported in this thesis (chapter 3 and 4), suggests that genetic variations which are associated with HAS1 aberrant splicing undergo mutational selection events that leave a mutational "trace" throughout the hematopoietic cell lineage, including tumor cells.

Thus, of three types of genetic variations identified in MM and WM patients, detection of germline origin HAS1 GVs that characterize MM or in WM patients can be used as biomarkers of predispositions for these diseases. They are currently being investigated in a cohort of 500 MM patients. We predict that HAS1 GVs found throughout the hematopoietic lineage cells (B, PC, T, and hematopoietic progenitor cells from blood) will provide markers to identify the subset of MGUS patients most at risk of transformation to overt malignancy. Those GVs categorized as tumor specific are predicted to provide valuable biomarkers for detecting early stages of overt malignancy, for evaluating tumor burden at the time of diagnosis, and for monitoring the response to therapy on all compartments of the MM or WM clone, not just those populations that are morphologically detectable.

C. 5. 7. Significance of the study

The work presented in this thesis includes several fundamental findings which offer new insights into the oncogenic events that underlie the biology of MM and WM. In addition, some novel possibilities for early identification of these diseases is suggested. A summary of the significance of the work follows:

1. The observations reported in Chapter 2 suggest that HAS1 novel variants (HAS1Va, HAS1Vb, and HAS1Vc) are upregulated in the circulating components of the myeloma clone and are biologically relevant markers of a circulating tumor burden. HAS1 variants are the first prognostic markers to be described for the circulating compartment of the malignant clone in MM. Furthermore, findings reported in Chapter 2 suggest that HAS1 and its novel variants are involved in the oncogenic process, in particular, by contributing to the spread of MM. Also, this study identified the HASVb intronic variant as clinically important in the biology of MM. Additionally, the work included in Chapter 2 supports the idea that intronic splicing of the HAS1gene is an early event that contributes to the genesis of MM and likely WM.

2. The observations reported in Chapter 2 also identified a possible source of intracellular HA. This fact has been one of the dilemmas in the field of Hyaluronan research since 1990s. Identification of intracellular source of HA i.e. intracellular HAS provides researchers with new tools to understand the exact function of intracellular HA through genetic manipulation of intracellular HAS.

3. The observations reported in Chapters 3 and 4 identified germline mutations

which are candidate predisposing markers for MM and WM and which may be useful to identify individuals at risk of developing MM or WM. These mutations can be used as predisposing markers for these diseases.

4. The observations reported in Chapters 3 and 4 identified genetic variations of hematopoietic origin which can be used to identify those individuals who have progressed further along the path towards overt malignancy but may not yet have undergone malignant transformation. As discussed above, there are no markers to distinguish which MGUS patients are at greatest risk for MM or WM. The hematopoietic-origin HAS1 GVs may be valuable markers for effective stratification of MGUS to identify those individuals with the greatest risk of transformation to overt MM or WM. Identification of high risk MGUS may then facilitate the development of preventive treatment strategies. Thus, these GVs are ideal for monitoring the events that may lead to generation of the earliest stages of malignant cells as they progress towards clinically overt malignancy.

5. The observations reported in Chapter 3 and 4 also identified tumor specific genetic variations, which provide markers for the identification of malignant clones in patients and can be used for early diagnosis and/or disease monitoring throughout the treatment of the patients. Furthermore, the tumor specific HAS1 GVs are likely to identify malignant cells that are clinically cryptic, including those that comprise the earliest stages of malignant transformation in MM or WM.

In summary, testing for germline and hematopoietic origin, and tumor specific genetic variations provides a testing sequence for increasing predisposition to disease and for use as an early maker of emerging malignancy. Using germline origin GVs will allow identification individuals "at risk" to develop the disease. In followup, these individuals can be monitored for premalignant stages of emerging disease by screening for hematopoietic origin GVs. However, for those patients who are already diagnosed with MM or WM, the pattern of HAS1 tumor-specific GVs will provide a sensitive "signature" to detect emergent malignant cells, assess tumor burden in the blood, evaluate the extent of response towards novel therapies during treatment, and predict imminent relapse.

The work presented in this thesis investigates the molecular basis of MM and WM. Understanding the mechanism(s) whereby the molecular events described here influence disease in MGUS, MM and WM may enable identification of new therapeutic targets and development of new therapeutic approaches to control MM and WM. Furthermore, the work presented here supports a novel idea that initial genetic changes that lead to MM are either inherited or are somatically acquired during the earliest stages of hematopoiesis. The involvement of hematopoietic progenitor cell populations in premalignant events preceding the development of overt MM and WM suggests mechanisms of oncogenesis that are similar to those underlying some leukemias, as discussed earlier. Finally, identifying new disease targets will ultimately yield development of new approaches to fight MM and WM.

C. 5. 8. References

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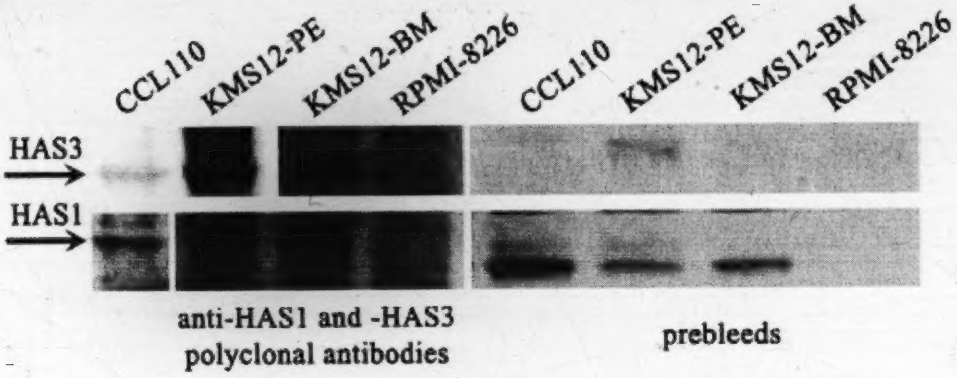
Supplemental materials for Chapter 2

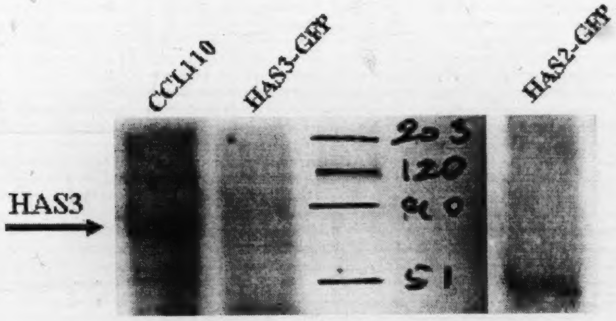
The 58 MM patients included in the cohort presented on Figure 2-3 had a median life expectancy of 413 days, far less than the median survival of 3.4 years seen in our patients locally and far less than would be expected of MM patients in general. The reason for this not clear, since the patients were not consciously chosen based on factors associated with poor survival. Further work would be required to show that the adverse prognostic significance of HAS1 splice variants seen in the reported cohort applies to myeloma patients with a better prognosis. However, work described in Chapter 4 suggests that the HAS1Vb is an excellent indicator of a subpopulation with exceedingly poor prognosis even among a larger cohort of 146 patients that in aggregate has a survival outcome of nearly 3 years. It worth noting that the poor outcome for the 58 patient cohort would have been expected to bias against our ability to detect clinically significant reductions in survival, indicating that even among a group of patients with very poor survival, HAS1Vb still stands out as an indicator of the worst prognosis.

Supplemental Figure 1 for Chapter 2. Western blotting



Expression of HAS transcripts (RT-PCR)

Cell lines	HAS1	HAS3
CCL110	+	+
KMS12-PE	+	+
KMS12-BM	+	+
RPMI-8226	—	—



HAS2- and HAS3-GFP construct were transfected into the COS cells

The HAS1 peptide used to raise HAS1 antibodies shares 8 aa and 9 aa similarities with similar regions of HAS2 and HAS3, respectively. This alignment is carried out manually, not considering the sequences upstream or downstream of the peptide stretch

```
HAS1- GNRAEDLYMVDM  FRF          HAS1- GNRAE DLYMVDM FRF

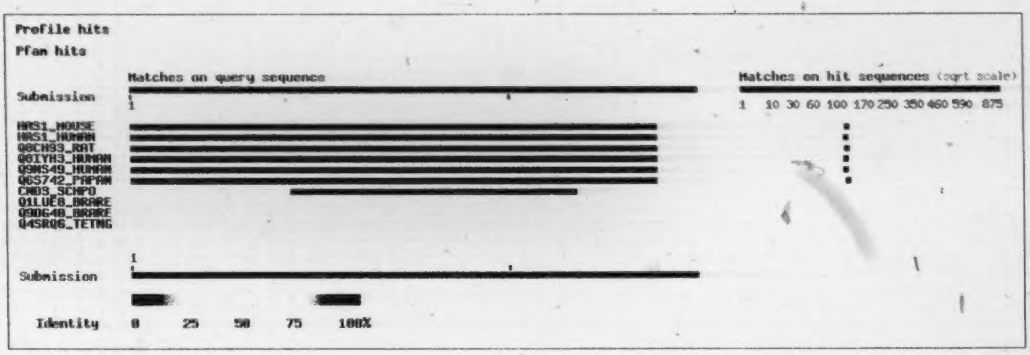
HAS2- GNSEDDLYMMDI FSE           HAS3- GNRQEDAYMLDIFHE
```

Even though there are ~50% similarities between these sequences BLAST analysis of HAS1 peptide against human genome demonstrated that HAS1 peptide used to raise antibodies shares significant similarities and identities with HAS1 sequences obtained from different species. This peptide did not align with any HAS2 sequence, and alignment with HAS3 was not significant.

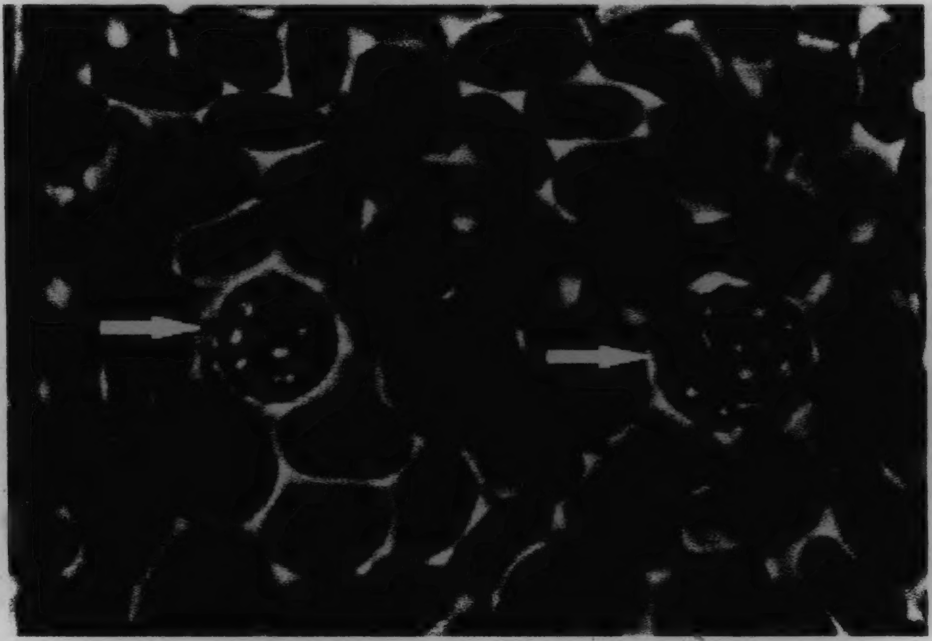
```
 Db AC      Description                                                 Score E-value

sp Q61647 HAS1_MOUSE Hyaluronan synthase 1 (EC 2.4.1.212) (Hyalu...   52  3e-06
sp Q92839 HAS1_HUMAN Hyaluronan synthase 1 (EC 2.4.1.212) (Hyalu...   52  3e-06
tr Q8CH93 _RAT Hyaluronan synthase 1 [LOC282821] [Rattus norvegic... 52  3e-06
tr Q8IYH3 _HUMAN Hyaluronan synthase 1 [HAS1] [Homo sapiens (Human)] 52  3e-06
tr Q9NS49 _HUMAN Hyaluronan synthase 1 [HAS1] [Homo sapiens (Human)] 52  3e-06
tr Q6S742 _PAPAN Hyaluronan synthase [HAS1] [Papio anubis (Olive ... 49  3e-05
sp Q10429 CND3_SCHPO Condensin complex subunit 3 (p100) (CAPG ho...   30    8.2
tr Q1LUE8 _BRARE Hyaluronan synthase 3 [has3] [Brachydanio rerio ... 30    8.2
tr Q9DG40 _BRARE Hyaluronan synthase 3 [has3] [Brachydanio rerio ... 30    8.2
tr Q4SRQ6 _TETNG Chromosome undetermined SCAF14503, whole genome ... 30    8.2
```

Graphical view of the alignment

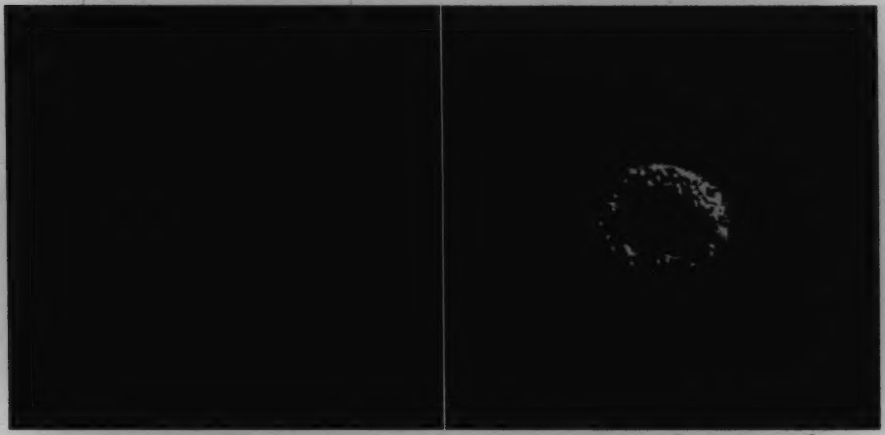


Supplemental Figure 2 for Chapter 2. B cells obtained from healthy donor peripheral blood (Particular Exclusion Assay)



B cells obtained from peripheral blood of healthy donor were cultured for 24 hours prior to particle exclusion assay. Arrows on the Figure donate peripheral blood B cells from healthy donor.

Supplemental Figure 3 for Chapter 2. Intracellular HA staining



MM CD19^{+}B cells were cultured for 48 hours, fixed, permeabilized and stained with B-HABP. HAS1 splice variant expression analysis demonstrated that these cells did not express HAS1Vb variant transcripts.